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II. CHEMICA

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THE CONTENT OF B-VITAMINS IN THE MILK OF COWS FED PURIFIED OR LOW-PROTEIN FEED, WITH UREA AS THE SOLE OR MAIN NITROGEN SOURCE, AND EVALUATION OF THE MICROBIOLOGICAL ASSAY METHODS

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SUOMALAINEN TIEDEAKATEMIA

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Communicated 14 February 1969 by A. I. VIRTANEN and J. K. MIETTINEN

PREFACE

The present work was carried out in the Laboratory of the Foundation for Chemical Research, Biochemical Research Institute, Helsinki, during the years 1963—68.

I am happy to have the opportunity of expressing my sincerest gratitude to my supervisor, Professor ARTTURI I. VIRTANEN, director of the Biochemical Research Institute, for his never failing interest, invaluable advice and constructive critisism in the discussions throughout the course of this work.

I also want to thank my teacher, Professor PAAVO ROINE, University of Helsinki, for valuable discussion and advice on the completion of the manuscript.

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The work was facilitated by the skilled technical assistance of Miss MARJA-LEENA AURA, Mrs. ANNA-LIISA PYYKKÖ and Miss MERI LUNDELL; I want to thank them for this help.

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Helsinki, February 1969.

MAIJA SAARIVIRTA

Contents

I. INTRODUCTION

Nutrition has a vital role in the health of man and influences socio-economic and cultural development profoundly. Malnutrition leads to deterioration of physical fitness and mental efficiency, and to reduction in the capacity to perform work. Global statistical surveys, based upon total food production, suggest that there is no world-wide shortage of food in terms of quantity or quality at the moment. But in the developing countries, where 2/3 of the world's population live, under-nutrition (too few calories) and malnutrition (especially lack of protein) are very common among people. In many areas, vitamin and mineral deficiencies are prevalent and foods furnishing these nutrients should be made available.

If the world's population continues to increase at 1965 rates, 50% more calories for the population will be required in 1985. In developing countries the need of more calories will be nearly 150%. With 30% reduction in fertility the increased caloric need in developing countries will still be 90—100%.[166]

One of the aids suggested for solving the food problem is to increase and improve domestic animal production, especially that of cattle which are able to make use of different kinds of feed material (for instance by-products of wood and sugar industries) useless to other mammals, and thus do not compete for food suitable for human consumption[210,212].

In the Biochemical Research Institute, Helsinki, Professor A. I. Virtanen, with his co-workers, has investigated the maintenance of milking cows on a protein-free feed (O-feed[1])) comprised mainly of briquets containing potato starch, purest sulphite cellulose and granulated sucrose as energy nutrients and urea and ammonium salts as the sole nitrogen source) during a period of six years. For about two years, feeding experiments with urea and different fodder-combinations low in protein (ULP-feed[1])) have been performed. The composition and yield of the experimental milks (O- and ULP-milks[1])) have been quite similar to those of milk (NorP-milk[1])) from cows fed rations common in Finland (NorP-feed: hay, silage, cereals and roots)[210,211,212].

Much consideration has been given to the question of the extent to which the minor constituents of milk, such as flavour substances and B-vitamins, are formed by ruminal biosynthesis or originate from the feed.

The synthesis of B-vitamins in the rumen has been demonstrated with low-vitamin diets[113,137,138,219]. However, in these experiments the low-vitamin diet consisted of mixed unpurified natural materials which, though poor in vitamins, certainly contained numerous compounds which the rumen population could use to synthesize B-vitamins.

The milk produced by the O-cows[1]) offers an opportunity to study whether the extent of the biosynthesis of B-vitamins in the rumen is adequate to maintain a normal B-vitamin concentration in the milk of the cow.

In this research the content of B-vitamins of O- and ULP-milks was investigated and compared with that of NorP-milk. The vitamins investigated were thiamine, riboflavin, nicotinic acid, pantothenic acid, pyridoxine, biotin, folic acid and cobalamin.

The survey of the literature, though comprehensive, is far from complete and many publications have been omitted to prevent further enlargement of the survey.

II. SURVEY OF THE LITERATURE

A. Structure, function and occurrence of B-vitamins

The B-vitamin complex is a heterogenous group of water-soluble compounds of low molecular weight which in their active forms function as coenzymes in biochemical reactions. The members of the group are ubiquitous in nature. Plants and most micro-organisms are generally able to synthesize them. For animals and some micro-organisms an external source of B-vitamins is necessary; there is evidence that microbial synthesis of the B-vitamins occurs in the rumen and thus ruminants are considered to be largely independent of an exogenous supply. However, the very young ruminant is dependent on such a source of vitamins before its rumen has fully developed[113].

In the large intestine of animals including man, micro-organisms synthesize B-vitamins but it is questionable whether the host, if not copro-

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1) O-feed = rations in which the only nitrogen source was non-protein nitrogen
O-cows = cows receiving the O-feed
O-milk = milk of O-cows
ULP-feed = urea-rich, low-protein rations
ULP-cows = cows receiving ULP-feed
ULP-milk = milk of ULP-cows
NorP-feed = normal protein-rich rations
NorP-cows = cows receiving NorP-feed
NorP-milk = milk of NorP-cows.

fagous, can use them in any significant extent. The enterically-synthesized vitamins are frequently bound inside the bacterial cells, or if secreted from the cells, are usually bound to protein; besides, the absorption of nutrients from the large intestine is relatively poor[4,35,92,110].

1. Thiamine

Thiamine functions in the form of thiamine diphosphate (thiamine pyrophosphate, TPP or cocarboxylase) in systems catalyzing either decarboxylations of α-keto acids or cleavage of α-hydroxyketones or α-diketones. TPP forms intermediates, which have been named »active acetaldehyde», »active pyruvate» and »active glycol aldehyde» in these reactions[25,83,84,141].

In plants thiamine is mostly in the free state, whereas in animals the principal form is thiamine diphosphate, though also the mono- and polyphosphates and thiamine disulphide have been isolated[16,97].

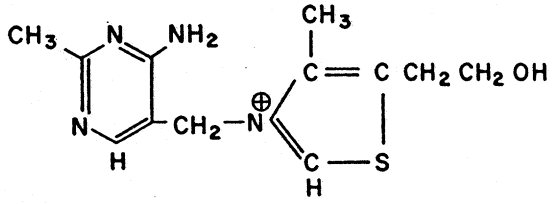

Fig. 1. Thiamine.

In cow's colostrum thiamine is present in free, phosphorylated and protein-bound forms[85,100]. In colostrum about 80% of the thiamine is present as TPP whereas in mid and late lactation milk hardly any TPP is found. The total amount of thiamine is 60—100 μg/100 ml colostrum, up to 60 μg in early and about 30—40 μg in mid lactation milk respectively. About 10% of the protein-bound thiamine is firmly bound to protein. The thiamine content of milk is independent of the nutrition of the cow[85]. According to Halliday *et al.*[68] the average free and total thiamine contents of the milk of Holstein cows are 23.4 and 40.5 μg/100 ml respectively, thus approximately 60% of the thiamine is present in the free state. Human milk is a relatively poor source of thiamine (16 μg/100 ml)[130].

2. Riboflavin

Riboflavin serves as a component in the oxidation-reduction systems in cells. Its functional forms are the phosphorylated flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD) which, bound to different

proteins, form the flavoproteins. The structure of riboflavin as a whole is reminiscent of that of the nucleosides. However, the ribose side-chain is not bound through a glycosidic linkage as in true nucleosides but is present as a pentitol. On the same basis FMN and FAD are not true nucleotides, but the designation nucleotide, though incorrect, has been generally accepted for them. The principal substrates which are dehydrogenated by flavoproteins are pyridine nucleotides, α-amino acids, α-hydroxy acids, aldehydes, purines and substances in which saturated carbon-carbon bonds are converted into conjugated ethylenic bonds[18,20].

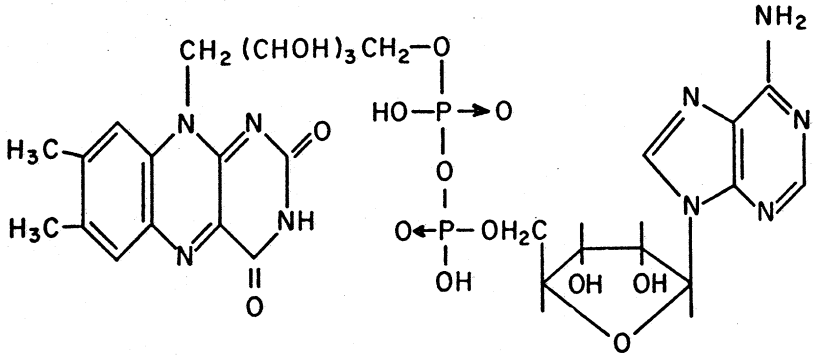

Fig. 2. Flavin-adenine dinucleotide (FAD).

In natural materials riboflavin is seldom present as the free vitamin. However, free riboflavin has been found as a major form of flavin in the milk of some species[134,144], urine[48], bull semen[221], the retina of fish[47], the venom of some species of snakes and in frogs' eyes and skin[18]. Of the nucleotides FAD is the most abundant (60—90%) form in nature. Most flavoproteins contain FAD as the coenzyme component[18].

The total riboflavin content of cows' milk recorded in literature ranges from 86 to 300 μg/100 ml[40,48,78,145,184,209]. About 10% of the riboflavin in milk is present in a form inactive for *Lactobacillus casei*[78]. According to Manson *et al.*[134] the ultrafiltrate of cows' milk contains free riboflavin and a small amount of FMN but no FAD at all. However, some FAD is found after heat treatment (3 min. 95°C). Modi *et al.*[144] observed a large riboflavin spot and a small FAD spot on paper chromatograms of extracts of cows', goats', ewes' and rabbits' milk, whereas with human, mares' and sows' milk only the FAD spot was visible. Japanese workers found in mid-lactation milk 55% free riboflavin, 29% FMN and 18% FAD of a total of 170 μg/100 ml; colostrum contained more FMN and FAD than did milk[147]. The average total riboflavin content of mature human milk is about 43 μg/100 ml[130]. A small amount of riboflavin in milk is in the fat-globule

membrane, over 90% of which is FAD[201]. The riboflavin content of the colostrum is usually higher than that of the milk[61,111,112].

The riboflavin content of milk can be influenced by the feed only to a limited extent[99]. Virtanen *et al.*[209] have shown that the riboflavin content of the milk of indoor-fed cows on AIV-forage is the same as that in the milk of grazing cows. After Hartman *et al.*[70] any effect of alteration in the feed is an indirect one, mediated through the rumen microflora.

3. Nicotinic acid

Nicotinic acid acts as cofactor in oxidation-reduction systems of cells. The functional forms are nicotinamide adenine dinucleotide (NAD, DPN, coenzyme I), nicotinamide adenine dinucleotide phosphate (NADP, TPN, coenzyme II) and nicotinamide mononucleotide phosphate (NMNP, coenzyme III). Among the reactions in which the first two cofactors (NAD and NADP) participate are the synthesis of high-energy bonds, glycolysis, pyruvate, pentose, lipid and nitrogen metabolism and photosynthesis[20,88,102]. NMNP acts as the coenzyme of yeast cysteinesulphinic dehydrogenase and is also the prosthetic group of dehydrogenases of animal origin[180,181].

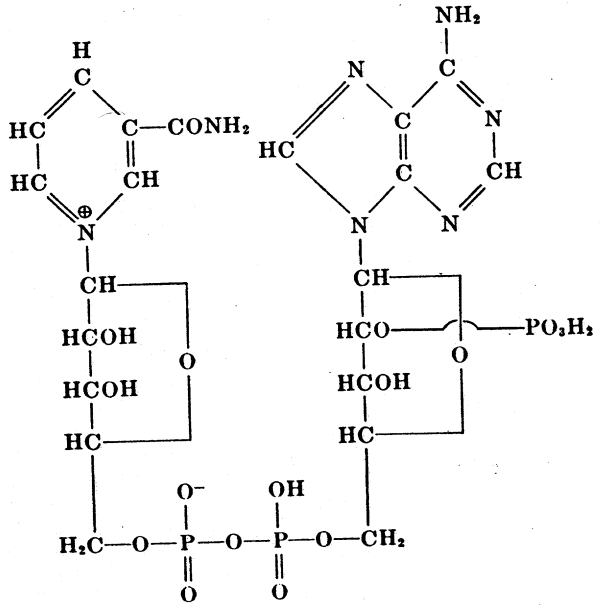

Fig. 3. Nicotinamide adenine dinucleotide phosphate (NADP).

Animal tissues and milk contain nearly all of the total activity as nicotinamide, while plants contain much less and a more variable amount of the total activity as nicotinamide[31,116]. Milk contains relatively little nicotinic acid[116]. However, since nicotinic acid is present in free form its absorbability is good. In addition, milk contains tryptophan (about 50 mg/100 ml) which can partly replace nicotinic acid in the diet[70]. It has been calculated that 60 mg of tryptophan is equivalent to 1 mg of nicotinic acid for man[59]. The nicotinic acid content of cows' milk ranges from 30 to 200 μg/100 ml and of human milk is around 170 μg/100 ml[61,70,130]. No significant difference has been found in the nicotinic acid content of the colostrum and milk of the cow[160].

There is little evidence of a direct effect of the composition of the feed on the nicotinic acid content of milk, though some authors report higher nicotinic acid content in the milk during outdoor feeding than during indoor feeding[61,123]. Considerable variation of the nicotinic acid content in milk of individual cows has been noticed but the day-to-day variations in the milk of any one cow were not very marked[123].

4. Pantothenic acid

Pantothenic acid is physiologically active as part of coenzyme A (CoA), the molecule of which contains also thioethanolamine (β-mercaptoethylamine), ribose, phosphoric acid and adenine. CoA functions in the transfer of acyl groups in the metabolism of proteins, carbohydrates, fatty acids, carotenoids, steroids and numerous other compounds[95,125]. In recent years another pantothenic acid coenzyme has been found, the acylcarrier protein (ACP) which functions in the biosynthesis of fatty acids. This coenzyme, 4′-phosphopantetheine, is made up of pantothenic acid, thioethanolamine and phosphoric acid and thus represents part of the CoA molecule[131,132,167]. Brown[26] found significant amounts of bound pantothenic acid other than CoA in micro-organisms and animal tissues; rat liver acetone powder contained 36.5 mμmoles free pantothenic acid, 103 phosphopantothenic acid, 6.8 pantetheine, 148 phosphopantetheine and 1003 CoA plus dephospho CoA/g.

Earlier, pantothenic acid was considered to be almost entirely in the free form in milk[61,75,123,160,192]. These results were probably due to inadequate extraction procedures. Later it has been demonstrated that about 25% of pantothenic acid in milk is in a bound form[70]. Recently Hibbitt[74] reported a production of 30 to 50 mg CoA/day in milk of cows. The concentrations of pantothenic acid reported in the literature range from 190 to 420 μg/100 ml cows' milk and from 80 to 350 μg/100 ml human milk[61]. The pantothenic

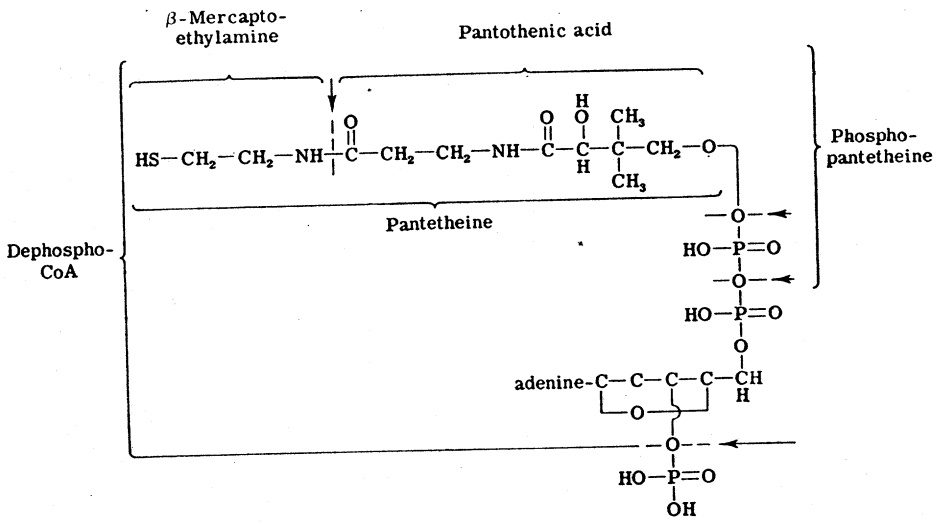

Fig. 4. Structure of coenzyme A and enzymatic degradation products[196].

acid content of milk varies from cow to cow and the day-to-day variation in the milk of a single cow is about 10%[123]. There seems to be no correlation between the feed of the cow and the pantothenic acid content of its milk[123,192]. Feeding 2 g pantothenic acid daily to cows resulted in no significant increase of pantothenic acid content in milk[135]. The pantothenic acid and biotin concentrations in milk are positively correlated[123].

5. Pyridoxine

Pyridoxine occurs in nature in three basic forms: pyridoxol, pyridoxal and pyridoxamine. The functional form of pyridoxine is pyridoxal phosphate. Studies of model reactions indicate that the pyridine ring, the phenolic group and the formyl group are necessary for all pyridoxine-dependent enzyme systems. Transformation of pyridoxal, pyridoxol and pyridoxamine to pyridoxal phosphate can occur by a number of different routes, all of which involve action of pyridoxal kinase[190]. Pyridoxal phosphate acts as a coenzyme in numerous reactions, *e.g.* transamination, racemization and decarboxylation of α-amino acids, oxidative deamination, α,β-cleavage of hydroxy amino acids and different elimination reactions[24].

Pyridoxal and pyridoxamine phosphates are the predominant forms of pyridoxine which occur in animal tissues and yeast; evidence for the pre-

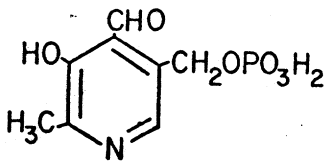

Fig. 5. Pyridoxal phosphate.

sence of pyridoxol phosphate is slight. In plants pyridoxol has been found in considerable amounts[123,168,171].

Gregory[64] found that pyridoxal accounted for about 80% of the total pyridoxine activity in milk, 20% being due to pyridoxamine. Rabinowitz *et al.*[170] found 32 μg pyridoxal and 9 μg pyridoxamine/100 ml milk; no pyridoxol was found. In milk powder Toepfer *et al.*[204] detected 3—4% pyridoxol, 30% pyridoxamine and 66% pyridoxal in a total of 2.7 μg/g. Siegel *et al.*[178] found 51 μg pyridoxine/100 ml milk of which 14% was in bound form. Gregory *et al.*[65] have shown electrophoretically the presence of small amounts of pyridoxamine phosphate in milk; evidence for the presence of pyridoxal phosphate was inconclusive. In fresh cows' milk the level of pyridoxine ranges from 9 to 60 μg/100 ml[30,63,224,228]. The individual variations of pyridoxine content in cows' milk as well as the variations in the milk of a single cow are 10—20%[63,103]. In human milk the average pyridoxine content is about 11 μg/100 ml[130].

6. Biotin

Biotin is present in natural materials in very low concentrations as free biotin, biocytin (biotinyllysine) and biotinsulphoxide[67,139,225]. The functional forms of biotin seem to be the carboxylated biocytin[118,127,223] and biotinyladenylate[179,207]. Biotin participates in carboxylation reactions, especially in the biosynthesis of fatty acids[53,215,216,217].

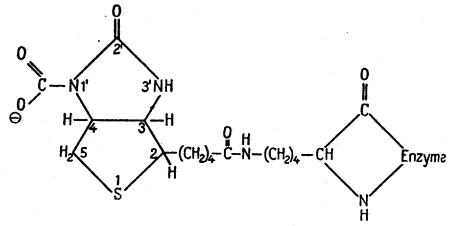

Fig. 6. N′-carboxybiotin coenzyme[223].

In biological materials biotin is present chiefly in conjugated and/or protein-bound forms[67]. However, in plants it is present partly in the free state[17]. It is claimed that biotin in milk occurs in the free form[76,78,117,123,192]. Many investigators have, however, used acid extraction to liberate »bound biotin» from milk[30,63,177]. The biotin contents in milk range from 0.2 to 8.4 μg/100 ml cows' milk and from a trace to 3.3 μg/100 ml human milk[61]. Lawrence *et al.*[123] found that the concentration of biotin in milk of different cows varied from 1.1 to 3.7 μg/100 ml and the day-to-day variations in the milk of one cow were from 2.1 to 4.0 μg/100 ml. Other investigators also have reported great variations in individual milks[78,192]. Stefaniak *et al.*[192] suggested this to be due to changes in composition of ruminal flora on different diets.

7. Folic acid

Folic acid or folate is the name given to 1. a group of compounds the structure of which is based on pteroic acid (PA) and 2. pteroylglutamic acid (PGA), the first known form of folate. PA is composed of a pteridin ring and *p*-aminobenzoic acid. The simplest folate member is PGA in which a glutamic acid residue is bound to the *p*-aminobenzoic acid of PA. This glutamic acid may be conjugated to one or more glutamic acid residues. The highest known pteroylglutamate contains six additional glutamic acid residues. All pteroylglutamates may exist as reduced di- or tetrahydroderivatives and/or may be substituted at the N^5- or N^{10}-positions with formyl, hydroxymethyl, methyl or formimino groups, or else the N^5 and N^{10} are linked through methylene or methenyl groups.

Folate functions as the carrier of the above mentioned groups in the biosynthesis of purines and pyrimidines and in the metabolism of certain amino acids, for example methionine, serine, valine and histidine. The exact nature of the coenzyme-form(s) of folate in natural materials has not

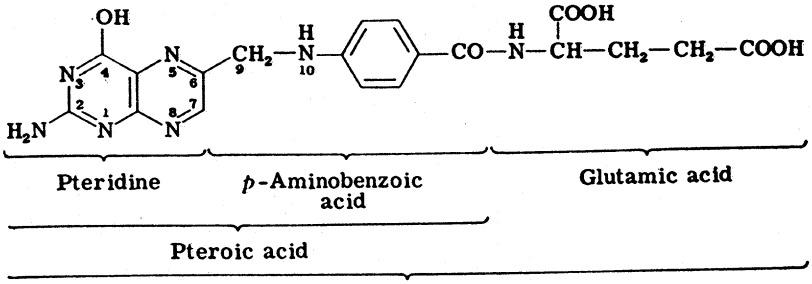

Fig. 7. Folic acid (Pteroylglutamic acid)[196].

been established. Present evidence indicates that the predominant forms of folate in natural materials are the reduced folates rather than the oxidized ones and therefore the former are considered to be the »active» forms[73,86,87,96,126152,172,206].

The concentration of folate in cows' milk given in earlier reports was minute. Collins *et al.*[36] estimated milk folate with *Streptococcus faecalis* and *Pediococcus cerevisiae* and found values from 0.06 to 0.60 μg/100 ml of which about 20% was in reduced form. Karlin,[103] using the same assay organisms, found 0.24 μg folic acid and 0.15 μg folinic acid (N^5-formyl-tetrahydrofolate, the most stable form of reduced folates) in 100 ml milk. After the discovery of the labile folates and subsequent reinvestigation of milk, the concentration of folate as measured with *L.casei* was found to be relatively high, namely from 3.7 to 12.4 μg/100 ml cows' milk[57,136,148,200]. Collins *et al.*[36] and Karlin[103] found several times more folate in colostrum than in milk whereas Ramasastri[173] found 4 times less in colostrum than in milk. The folate content of human milk varies from 0.74 to 6.10 μg/100 ml[104,136,173]. All assays reported for human milk have been made with *L. casei*, which responds to N^5-methylfolates whereas *S. faecalis* and *P. cerevisiae* do not. Individual variations of folate content in both human and cows' milk are considerable[104].

8. Cobalamin

Cobalamin is the name of a group of compounds which contain a porphyrin-like corrin nucleus with a central cobalt atom. The compound earlier known as vitamin B_{12} (cyanocobalamin) is now named α-(5,6-dimethylbenzimididazolyl) cobamide cyanide, though the trivial names cyanocobalamin and vitamin B_{12} are still permissible. Some of the other members of cobalamins are: α-(5,6-dimethylbenzimidazolyl) aquocobamide and -hydroxocobamide (aquocobalamin and hydroxocobalamin), α-(2-methyladenyl)cobamide cyanide (Factor A) and α-(adenyl)cobamide (pseudovitamin B_{12}). The coenzyme-forms of vitamin B_{12}, in all of which an organic ligand is attached to the central cobalt atom, are called cobamides or cobalamins, *e.g.* the coenzyme derived from cyanocobalamin is 5′-deoxyadenosylcobalamin or α-(5,6-dimethylbenzimidazolyl)-Co-5′-deoxyadenosylcobamide[19,93].

The mode of action of cobamide coenzymes is only partly understood; difficulties arise from the diversity of reactions in which these coenzymes participate. Some of the enzymes with cobamide coenzymes are: glutamate mutase, methylmalonyl-CoA-mutase, dioldehydrase, ethanolamine deaminase and ribonucleotide reductase[5,214]. Pseudovitamin B_{12}, although known

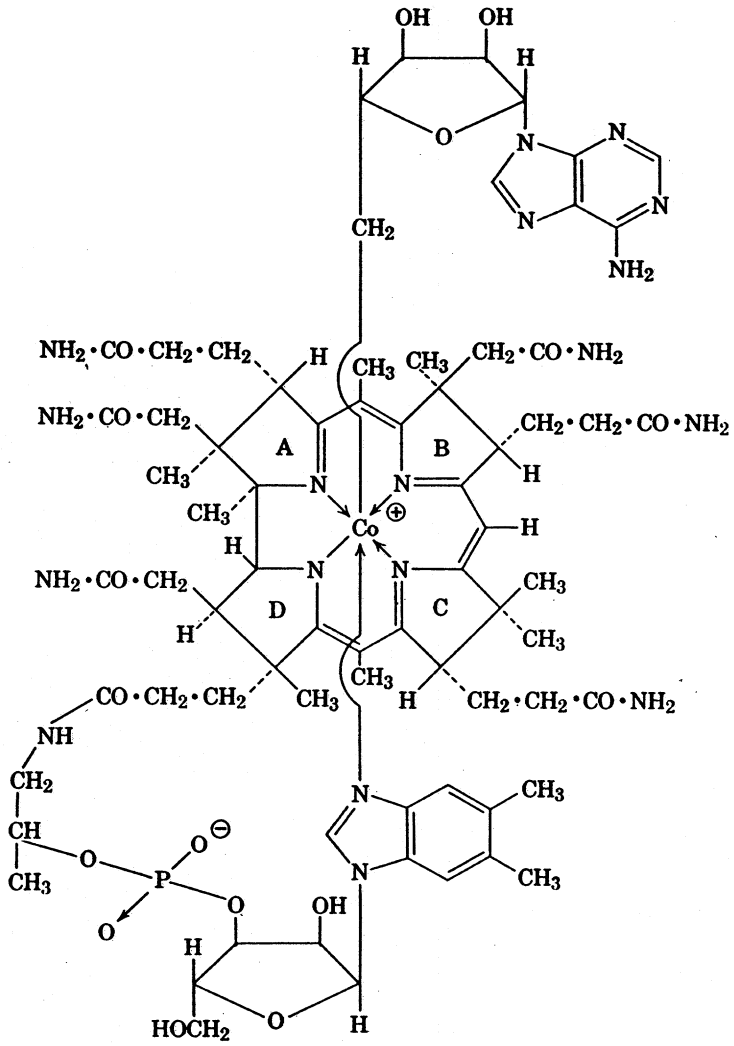

Fig. 8. Vitamin B_{12} coenzyme (5′-deoxyadenosylcobalamin)[19].

to be clinically inactive, is a coenzyme in glutamate metabolism in some microbial systems[124].

It seems quite certain that the primary source of cobalamin and its analogues in nature is microbial synthesis[70]. In natural materials cobalamin is almost entirely present as coenzymes; cyano- and hydroxocobalamin previously isolated were artifacts produced by chemical decomposition (effects of light, heat, cyanide) during isolation. In ethanol extracts of human, rabbit, sheep and chicken liver 48—72% of the cobalamin was shown to

be in the form of coenzymes[195,205,220]. Whether cobalamin exists in higher plants is still questionable[46]. However, cobalamin analogues are present in certain fermented fodders and silage[70].

In rumen contents, vitamin B_{12} itself makes up about 10% the activity for *E.coli*, Factor A (2-methyladenyl cobamide) comprises about 60%, while pseudovitamin B_{12} (adenyl cobamide), Factor B (no nucleotide) and Factor C (guanyl cobamide) account for most of the remainder[43]. Even though cobalamin analogues predominate over cobalamin in the alimentary tract of ruminants they occur in milk and animal tissues only in traces[70].

In cows' milk 95% of the cobalamin is bound to all protein fractions, the whey fraction containing more than the others[108]. About 70% of the bound cobalamin is bound to peptides of molecular weight 3000—9000[58]. The concentration of cobalamin in the milk of the cow ranges from 100 to 1450 mμg/100 ml[3,30,36,62,103,105,109,143,157]. The cobalamin activity of colostrum is 3—10 times higher than that of milk[3,36,103]. The variations of cobalamin levels in milk of different cows as well as in different samples of milk of the same cow are great[103,157]. The cobalamin content of human milk is about 30 mμg/100 ml[62].

B. B-vitamin requirements of man

The amount of B-vitamins required by man varies according to diet, activity, health, response to stress, body size, sex, age, *etc.* Therefore the daily intakes recommended are presented with a safety margin to maintain good health[34,14]. According to the Food and Nutrition Board[51] the recommended daily amounts of B-vitamins for adult persons are: thiamine 0.9—1.4 mg, riboflavin 1.5—1.7 mg, niacin equivivalent 10—18 mg, pyridoxine 2 mg, folic acid 0.4 mg and cobalamin 5—6 μg. The requirements for pantothenic acid and biotin are not specified.

C. Ruminal synthesis of B-vitamins

It is difficult to measure the total synthesis of B-vitamins in the rumen, since their concentration depends on the balance between the vitamin intake in the feed and microbial synthesis on the one hand and the removal by absorption from the rumen, passage to the intestine, utilization by micro-organisms and possible degradation on the other. However, comparison of the vitamin content of the rumen with that of the feed indicates that the microbial population of ruminants synthesizes thiamine, riboflavin, nicotinic acid, pantothenic acid, pyridoxine, biotin, folic acid and cob-

alamin[71,113,137,138,219]. Kon *et al.*[113] compared the vitamin content of feed with that of the rumen of steers four hours after feeding hay, hay and concentrates or a low-vitamin feed (casein and NaOH-treated straw) and found relatively little synthesis with the two hay feeds but considerable synthesis with the low-vitamin feed. The rumen vitamin concentration with all 3 feeds was broadly similar.

Lardinois *et al.*[120] observed that the addition of 200 g urea daily as additional nitrogen definitely increased rumen synthesis of riboflavin, nicotinic acid, biotin and pantothenic acid when a readily fermentable carbohydrate (corn molasses) was present in the feed. Pyridoxine and folate could not be correlated with ration composition. In the absence of a readily fermentable carbohydrate the ruminal synthesis of vitamins was not at a maximum.

Porter[165] has investigated the vitamin balance of lactating cows receiving high-concentrate/low-roughage feed. The vitamin content of the feed was unusually high. His results are given in Table 1.

Table 1

Mean value for the vitamin content of diet, rumen, urine, faeces and milk of two cows receiving a high-concentrate/low-roughage diet.[165]

Vitamin	Intake mg/24 h	Rumen total, 5 h after feeding, mg	Output		
			Urine mg/24 h	Feaces mg/24 h	Milk mg/24 h
Nicotinic acid	500	1900	90	130	8
Cobalamin	0	—	—	—	0.03
Biotin	4	28	0.03	0.2	1
Riboflavin	130	63	7.5	45	15
Pantothenic acid	120	50	45	10	90
Pyridoxine	38	24	3	27	4
Thiamine	27	30	—	20	3
Folic acid	7	3.1	1.5	3.5	—

The amounts of nicotinic acid and biotin were considerably greater in the rumen than in the feed, whereas that of thiamine was similar and those of other vitamins were less. Only with pantothenic acid and cobalamin was the total output greater than the intake from the feed. 75% of the pantothenic acid indigested was secreted in the milk. Cobalamin, which was absent from the feed, was found in the milk.

Nilson *et al.*[151] have studied the effect of ration change on rumen microorganisms and on the concentration of nicotinic acid and pyridoxine in

rumen fluid and milk. Each of the rations consisted of a single feed: 1. corn silage, 2. alfalfa hay and 3. grain concentrate mixture containing 12% crude protein. Before the test the cows received a mixture of these 3 feeds. The cows were adapted to the test rations by adding increasing amounts of one feed and restricting the amounts of the other two; the adaptation period was 4 days. The total microbial count decreased from 10^{10} to 10^{6} on the 5th day of experiment with all 3 feeds. After that the count slowly increased reaching its initial level on the 21st day. The nicotinic acid content in milk was similar for the grain and corn silage rations and with both the level increased from an initial value of 75 μg/100 ml to 100 μg/100 ml; the nicotinic acid content in milk was lowest with the alfalfa hay ration although the vitamin content of this ration (673 mg/day) was much greater than that of the other two (about 360 mg/day). The concentration of pyridoxine in milk with the alfalfa hay ration (35 μg/100 ml) was higher than that with the other two rations (19—28 μg/100 ml). The amount of nicotinic acid in the rumen was 380 μg/100 ml with the grain ration; it declined from 310 μg to 175 μg/100 ml on the 21st day with corn silage. The concentration of nicotinic acid in the rumen was lowest with the alfalfa hay ration. The amount of pyridoxine in the rumen was slightly higher with the grain ration than that with the other two rations.

Clifford *et al.*[33] found that when B-vitamins were given with the feed or injected into the animal they had no significant effect upon vitamin concentration in the rumen.

The opinion that feed can influence the vitamin content of the rumen and milk by changing the microbial flora of the rumen has been presented[67,89,174]. The protozoa do not synthesize B-vitamins[89] or synthesize only some of them[37].

Dryden *et al.*[43] investigated the production of cobalamin and its analogues *in vitro* by 48 strains of rumen bacteria. *Selenomonas ruminantium* and *Peptostreptococcus elsdenii* and to a lesser extent *Butyrofibrio fibrinosolvens* and an unidentified group »B 385-1» appeared to be the principal organisms responsible for the production of cobalamin and its analogues in the rumen of the cow.

Nurmikko[156] has studied *in vitro* the symbiotic relationships of lactic acid bacteria the nutrient requirements of which are relatively complex. He found that when different strains were cultured together, the medium could be much simpler than when each of the organisms was grown alone. One organism was able to secrete into the medium growth-factors (vitamins and amino acids) which the others needed but could not synthesize themselves, and *vice versa*.

The mean microbial counts in the rumen fluid of O-, ULP- and NorP-cows, determined in connection with the feeding experiments described

in the Introduction, are shown in Table 2. The number of bacteria in the rumen fluid of the O-cows was about 6 and 2.5 times as high as that of the NorP- and ULP-cows respectively. There were no protozoa in the rumen fluid of the O-cows, and the number of protozoa in the rumen fluid of the ULP-cows was about one third of that of the NorP-cows[146].

Table 2

The mean microbial counts in the rumen fluid of O-, ULP- and NorP-cows.[146]

Feed	Rumen contents	
	Protozoa, count/ml	Bacteria, count/ml
O	0	1.86×10^{10}
ULP	2.32×10^{4}	7.65×10^{9}
NorP	7.43×10^{4}	3.20×10^{9}

D. Estimation of B-vitamins

B-vitamins can be estimated by physical, chemical, biochemical, biological or microbiological methods. None of these methods, except the biological (animal) assays, is specific for one substance only, since many other compounds in the test material may have characteristics responding to the principle of the assay. The vitamins can, of cause be purified by solvent extraction and/or chromatographic methods. However, the assay becomes more elaborate, time consuming and unpractical for routine analyses of large number of samples. A great advantage of chromatographic purification is that with proper arrangement it is possible to obtain information about the different forms of the vitamin in the material[67,199].

1. Principle of microbiological assays

The principle of microbiological assays is to measure the response of the test organism to some essential nutrient in an otherwise complete growth medium. The nutritional requirements of the assay organism must be clearly defined, so that a medium completely free of the substance to be measured but containing all other factors necessary for optimal growth can be provided. Graded amounts of the standard vitamin or the test sample are added to the otherwise complete medium, which is then inoculated with the test organism; after incubation the growth is measured. The standard

curve is made by plotting the growth *versus* the amount of standard vitamin, and the vitamin concentration of the test sample is calculated from the figure obtained from the curve. Values obtained at different concentration levels of the unknown should agree within $\pm$ 10% of the mean.

2. Assay organisms and extraction methods

a. Thiamine

For *Saccharomyces cerevisiae* (ATCC 7753[2]) the pyrimidine component of thiamine is about 30% as active, the thiazole component 60% as active and TPP less than 3% as active as thiamine itself on an equimolar basis[142]. *Kloeckera brevis* (ATCC 9774) does not use the pyrimidine or thiazole components either together or alone: TPP is about 70% as active as free thiamine on a molar basis[80]. *Ochromonas malhamensis* (ATCC 11532) has the advantage of responding only to intact thiamine[9]. For *Lactobacillus fermenti* (ATCC 9338) TPP is about 30% as active as free thiamine on a molar basis. The pyrimidine and thiazole components are inactive if the incubation period is restricted to about 18 hours. After 48 hours of incubation their responses are about the same as that of thiamine. The main difficulty with *L. fermenti* as an assay organism is its tendency to develop, occasionally, the ability to synthesize thiamine. However, this can be overcome by adding high levels of thiamin to the stock culture medium[32,175]. For *Lactobacillus viridescens* (ATCC 12706) thiamine and TPP have about the same activity[28,41]. Several other organisms have been used for thiamine assays but do not seem to have gained general acceptance[115,142,143].

Thiamine is usually extracted by dilute acid hydrolysis followed by enzymatic digestion with a phosphorolytic enzyme. Sometimes it seems advisable to complete the liberation from proteins by digestion with some proteolytic enzyme[162]. Generally, the sample is treated with 0.1 N sulphuric acid or hydrochloric acid on a steambath for 30 minutes, or autoclaved 15 minutes at 121°C[142,162,182,189]. After heating the pH is adjusted to 4.5 and some phosphorolytic enzyme is added. The mixture is incubated at 45—50°C for 3 hours or at 37°C overnight[142,162].

b. Riboflavin

Lactobacillus casei (ATCC 7469) and *Leuconostoc mesenteroides* (ATCC 10,100) have been used for riboflavin assays. The response of *L. casei* to free riboflavin, FMN and FAD differs significantly, riboflavin having the

highest activity[119]. *L. casei* has the disadvantage that starch and some fatty acids stimulate its growth. However, starch can be removed from the test solution by diastase digestion, and fatty materials by filtration at pH 4.5[163]. *L. mesenteroides* is 50 times as sensitive as *L. casei*. It is also stimulated by fatty acids[114].

Although mere heating in a large volume of water is often sufficient to liberate riboflavin from bound forms in natural materials, it has been found in practice that more consistent and slightly higher values are obtained by heating with dilute mineral acid in an autoclave for 15 minutes at 121°C[189]. Riboflavin can also be liberated by treatment with 5% trichloroacetic acid at 37°C overnight. Under these conditions FAD is split to FMN, but the latter is not hydrolyzed further to free riboflavin[27]. Heating in an autoclave at 121°C for 15 minutes with subsequent digestion with either a phosphorolytic enzyme or with both phosphorolytic and proteolytic enzymes has also been used[40,163,218]. The results obtained with *L. casei* from such extracts were comparable with those obtained by a fluorometric method[40].

c. Nicotinic acid

Lactobacillus plantarum (ATCC 8014. earlier *L. arabinosus*), *Leuconostoc mesenteroides* (ATCC 10,100) and *Tetrahymena pyriformis* have been used to estimate nicotinic acid. *L. plantarum* responds equally well on a molar basis to nicotinic acid, nicotinamide, nicotinuric acid and NAD[134]. *L. mesenteroides* responds only to nicotinic acid[98]. *T. pyriformis* utilizes nicotinic acid and nicotinamide, but when they are added together at the same concentration the increment of growth is less than the sum of the increments obtained when the two forms are used separately[12].

The common method for extraction of nicotinic acid from natural materials is to autoclave the sample in a large volume of water or dilute mineral acid (1 N) for 1 hour at 121°C[185]. Krehl *et al.*[116] preferred the use of hydrochloric acid, since in their experiments they obtained 100% recovery with this acid whereas only 75% recovery was obtained with sulphuric acid extraction. Johnson *et al.*[98] recommended extraction with sulphuric acid, since according to their experience the neutralization of hydrochloric acid with NaOH increased the NaCl concentration to inhibitory levels. Lawrence *et al.*[123] used enzymatic digestion with polidase, mylase, clarase or takadiastase and obtained the same results as those with water or acid treatment. Holmes *et al.*[82] used papain-diastase digestion for 24 hours at 45°C.

d. Pantothenic acid

All micro-organisms tested require pantothenic acid for growth but only some of them have been used as assay organisms (Table 3). If asparagine is present in the assay medium *Saccharomyces carlsbergensis* (ATCC 9080) is specific for pantothenic acid and does not respond to other pantothenates[8,26,39]. For *Lactobacillus helveticus* (ATCC 12046) pantetheine and phosphopantetheine are 100 times more active than pantothenic acid; CoA is inactive[26,39]. *Lactobacillus casei* (ATCC 7469) responds equally well to panto-

Table 3

Activities of different test organisms to pantothenic acid and its derivatives[26,39,196].

Compound	*L. plant*	*L. cassei*	*L. helv.*	*L. bulg.*	*A. subox.*	*S. carlsb.*
Pantothenic acid	+	+	±[1]	±[1]	−	+
Phosphopantothenic acid	−	−	−	−	*	−
Pantetheine	+	+	+	+	*	−
Phosphopantetheine	−	*	+	*	+	−
Dephospho CoA	−	−	−	*	*	−
CoA	−	−	−	−	+	−

[1]) Active if present in high concentration. + active, − inactive, * no data.

thenic acid and pantetheine but not at all to CoA[39]; its response to phosphopantetheine is not known. For *Lactobacillus plantarum* (ATCC 8014) pantetheine has the same response as pantothenic acid if 10 mg of cysteine is added per 5 ml of medium[222]; without cysteine its activity is about 40% of that of pantothenic acid[39]. *Lactobacillus bulgaricus* is specific for pantetheine. *Acetobacter suboxydans* (ATCC 621 H) responds only to phosphopantetheine and CoA[196].

As pantothenic acid is very sensitive to any severe treatment with acid or alkali it is extracted from natural materials with water in neutral medium or by enzymatic digestion. For most materials enzyme treatment is necessary, since not all bound forms of pantothenic acid are available to the assay organisms[26 39,150,154]. Neal *et al.*[150] compared the biological chicken assay with microbiological assay of untreated and clarase digested yeast powder. The untreated sample gave 24% and the clarase treated sample 67% of the pantothenic acid activity obtained with the chicken assay. Treatment of the sample with alkaline intestinal phosphatase and crude chicken liver enzyme and subsequent assay with *L. plantarum* gave values comparable with those obtained with the chicken assay[101,154,155]. Brown[26] has presented a summary of the degradation of pantothenates by various

enzymes (Table 4). Several authors are of the opinion that pantothenic acid can be extracted from milk by autoclaving for 15 minutes at 121°C with a large amount of water, and that enzymatic digestion with takadiastase, papain, clarase or mylase does not increase the pantothenic acid yield[8,75,78,123,192]. Gregory [61] obtained a slightly higher concentration of pantothenic acid from milk treated with chicken liver enzyme and intestinal phosphatase than from untreated milk.

Table 4

Products formed from CoA and related compounds by treatment with various enzymes[26].

Substance treated	Treatment with			
	Prostate monophosphatase	Intestinal phosphatase	CoA pyrophosphatase	Avian liver enzyme
CoA	Dephospho-CoA	Pantetheine	Phospho-pantetheine	—
DephosphoCoA	—	Pantetheine	Phospho-pantetheine	—
Phosphopantetheine	—	—	—	Phosphopantothenic acid
Pantetheine	—	—	—	Pantothenic acid
Phosphopantothenic acid	Pantothenic acid	Pantothenic acid	—	—

e. Pyridoxine

Saccharomyces cerevisiae (ATCC 7752) responds to total pyridoxine[178] but the responses to different forms differ considerably[187]. The response of *Saccharomyces carlsbergensis* (ATCC 9080) to free and phosphorylated forms varies somewhat. Approximately the same activity of all free forms on a molar basis was reported[186,187], but later it was found that the organism does not respond to pyridoxamine to the same extent as to pyridoxal and pyridoxol[64,159,197]. According to Hodson[77], the response of *S. carlsbergensis* to the free and phosphorylated forms of pyridoxine is about the same, but Rabinowitz *et al.*[169] reported that the activity of the phosphorylated forms is much less than an equimolar amount of the free forms. Edwards *et al.*[44] criticized the wide variations in results obtained in different laboratories in a collaborative study using rats and *S. carlsbergensis* as pyridoxine assay organisms, and suggested a careful reinvestigation of the methods.

The response of *Neurospora sitophila* (ATCC 9276) to all free pyridoxines in the absence of thiamine is approximately equal. The values obtained from natural materials with *Neurospora* assays are in good agreement with those of animal assays of the same or similar materials[187,193]. Harris[69] suggested the addition of thiamine to the medium to eliminate the stimulating effect of thiamine present in the sample and to increase the sensitivity of the assay. Hodson[77] followed this suggestion but found that the response of the mould to pyridoxamine and to a lesser extent to its phosphate was decreased, the response to pyridoxol, pyridoxal and pyridoxal phosphate being unchanged. *Streptococcus faecalis* (ATCC 8043) responds to pyridoxamine and pyridoxal though the response to the latter is somewhat smaller; pyridoxol is inactive[64,168,186,187]. Of the phosphorylated forms pyridoxamine phosphate is from 0.7 to 2 times as active as pyridoxamine whereas pyridoxal phosphate is much less active than an equimolar amount of pyridoxal[168,169]. The response of *Streptococcus faecium* (Ø 51) to pyridoxamine and pyridoxal is the same as that of *S. faecalis* but the former is more sensitive in the sense that it does not respond to alanine[64], whereas *S. faecalis* does[188]. *Lactobacillus casei* (ATCC 7469) responds to pyridoxal, the corresponding phosphate being only slightly active[64,169,170,188]. With this organism pyridoxal can be replaced by *d*-alanine in the presence of *l*-alanine which alone has no effect[188]. The different responses of *S. carlsbergensis*, *S. faecalis* or *S. faecium* and *L.casei* to pyridoxines have been used in differential assays of pyridoxines[64]. *Tetrahymena pyriformis* responds almost equally as well to pyridoxamine, pyridoxal and pyridoxal phosphate; 120 times more pyridoxol is required to yield the same growth response. When pyridoxal and pyridoxamine are added together in the same concentration to the medium, the growth increment is less than the sum of the individual increments[12,13].

The complicated relationships of these assay organisms to the five known forms of pyridoxine (Table 5) necessitates some kind of hydrolytic treatment to liberate free pyridoxine from protein-bound and phosphorylated complexes. Enzymatic digestion, although not common, has been used. For example, Baker *et al.*[13] used clarase or diastase for assay of blood pyridoxine with *Tetrahymena*, and Gregory[65] used intestinal phosphatase in milk analysis. Atkin *et al.*[7] compared clarase digestion and acid hydrolysis in liberating free pyridoxine and found only slightly higher values with the latter method. For acid hydrolysis different concentrations, from 0.055 N to 2 N hydrochloric or sulphuric acids, pressures from 15 to 20 lb and periods of 30 min. to 5 hours have been used[7,178,193,204]. The use of large amounts of dilute acid, high pressure and a long period of heating was suggested by Rabinowitz *et al.*[169]. However, less dilute acid (3 N) and a short period of heating may sometimes be preferred to avoid possible rebinding of the liberated pyridoxine to proteins[198].

Table 5

Comparative responses of different assay organisms to pyridoxal, pyridoxol, pyridoxamine, pyridoxal phosphate and pyridoxamine phosphate.

Assay organism	Pyridoxol	Pyridoxal	Pyridoxamine	Pyridoxal phosphate	Pyridoxamine phosphate	Reference
S.cerevisiae	1.0	0.41–1.2	0.16–0.64			187
S.carlsbergensis	1.0	1.42	1.33			187
	1.0	0.9–1.4	0.8–1.3			186
	1.0	0.9	0.87	0.95	0.96	77
	1.0	1.06	0.63			64
Neurospora [1])	1.0	1.4	1.4			187
sitophila	1.0	1.13	0.30	1.06	0.55	77
Tetrahymena pyriformis	–	+	+	+		12
Streptococcus	1.0	5000–8000	6000–9000			186
faecalis	1.0	5500	8000			187
Lactobacillus	1.0	1000–1500	3–10			186
casei	1.0	1450	10			187
	inact.	1.0	inact.			64
Streptococcus faecium	inact.	0.87	1.0			64
White rats	1.0	1.2	1.6			187

[1]) Upper line: assay medium without thiamine, lower line: assay medium with excess of thiamine.

f. Biotin

Several micro-organisms have been used for biotin assays. Table 6 presents the response of some of them to the two biotins most common in natural materials.

Table 6

The response of some micro-organisms to biotin and biocytin.

Test organism	Biotin	Biocytin	Reference
L.plantarum	+	–	202
L.casei	+	+	202
S.cerevisiae	+	+	227
N.crassa	+	+	11
O.danica	+	+	1
M.sodonensis	+	++	199
A.boydii	+	+	

Lactobacillus plantarum (ATCC 8014) is quite specific to free biotin[202]; of the known derivatives of biotin only oxybiotin, biotinylglycine and biotinyl-*d*-sulphoxide can replace it[140,227]. However, it is not known whether these compounds occur naturally[67]. *Lactobacillus casei* (ATCC 7469) responds equally well to biotin and biocytin[202]; biotinyl-*d*-sulphoxide is inactive[140]. Biotinamide, biotinylglycine and biotinyl-β-alanine are among the synthetic biotin derivatives to which *L. casei* responds[226]. *Saccharomyces cerevisiae* (ATCC 4228) responds to both biotin and biocytin[202] and biotinyl-*d*-sulphoxide is as active as biotin[140]. *Ochromonas danica* responds to biotin and biocytin; desthiobiotin is an inhibiting compound[11]. With *Allescheria boydii* (ATCC 9258) biotin and biocytin are equally as active[199]. The organism is a pathogenic fungus which makes it less desirable as an assay organism[203]. For *Micrococcus sodonensis* (Nov.SP) biocytin is more active than biotin[1]. *Neurospora crassa* responds to biotin, biocytin and to several biotin derivatives and degradation products[227].

The methods used to extract biotin from natural materials differ somewhat. Drastic acid hydrolysis with 2 to 6 N sulphuric acid for 1 hour at 121°C has been recommended, especially when *L. plantarum* is the assay organism[21,49,203,225]. Many authors suggest autoclaving with water for extraction of biotin from milk, since the assay results in their experiments were not changed by treatment with acid or several enzymes[78,123,192,225]. Other authors have used 2 to 3 N sulphuric acid to extract biotin from milk[117,177].

g. Folic acid

Folate is present in natural materials in a number of coenzymatically active forms to which the response of different organisms varies greatly (Table 7). *Streptococcus faecalis* (ATCC 8043) responds to unconjugated folates except N^5-methyltetrahydrofolate[148,189]. It responds also to diglutamates[45,91] but does not grow well on some of them[72,194]. Pteroic acid, N^{10}-formylpteroic acid and N^5-formyltetrahydropteroic acid, which are inactive for the common assay organisms, are as active as folic acid for *S. faecalis*. These compounds are inactive for man[72,172]. *Lactobacillus casei* (ATCC 7469) responds to mono-, di- and triglutamates but not to the higher polyglutamates, and the reduced derivatives investigated are as active as pteroylglutamic acid[172,189,206]. *L. casei* is the only assay organism known to grow on N^5-methylfolates, the main folates in human serum and animal livers[72,121,152,159]. *Pediococcus cerevisiae* (ATCC 8081), earlier known as *Leuconostoc citrovorum*, requires reduced folates[169,171]; pteroic acid and its derivatives, N^5-methylfolates and higher polyglutamates are inactive[45,72,91]. With *Tetrahymena geleii* pteroic acid and its derivatives as well as N^5-methyl-

folates are inactive[45,91]; folic acid and all conjugated forms have approximately the same activity on a molar basis[107].

Since folates are destroyed by acid and alkali[189] enzymatic digestion must be used for extraction. Reduced folates, except for N^5-formyltetrahydrofolate, are oxygen labile and must be protected with ascorbic acid during extraction[15,54]. The conjugases, folate splitting enzymes, are widely distributed in tissues[22,23]. Some samples, *e.g.* liver, kidney, serum and plasma can be prepared for assay by autolysis in phosphate-ascorbate buffer[10,33,72]. Two types of conjugases have been used, one from chicken pancreas with a pH optimum of 7.5[106,122] which splits polyglutamates to the diglutamate level, and the other, present in hog kidney, the gas gland of *Physalia physalis* (a member of the hydrozoa) and rat liver with a pH optimum of 4.5. Polyglutamates are split to monoglutamates with these conjugases[23,152].

Table 7

Activity of folates for micro-organisms.

Folate	*S. faecalis*	*L. casei*	*P. cerev.*	*T.geleii*	Reference
PA[1])	+[2])	−	−	−	45, 72
N^{10}−CHO−PA	±	−	−	−	45, 189
N^5−CHO−PA−H_4	+	*	−	*	45, 91
PGA	+	+	−	+	45, 107, 107, 196
N^5−CH_2OH−PGA	+	+	*	*	45
N^{10}−CHO−PGA	+	+	−	*	45, 149
N^5−CH_3−PGA	−	+	−	−	149
PGA−H_2	+	+	+	*	45
N^{10}−CHO−PGA−H_2	+	+	−	*	45
N^5−CH_3−PGA−H_2	−	+	−	*	72
PGA−H_4	+	+	+	+	196
N^5−CHO−PGA−H_4	+	+	+	+	45, 91, 196
N^5−CHNH−PGA−H_4	+	+	+	*	45
N^5−CH_2OH−PGA−H_4	+	+	+	*	45
N^5N^{10}=CH−PGA−H_4	+	+	+	*	45, 196
N^5−CH_3−PGA−H_4	−	+	−	−	72, 196
PDGA	+	+	*	*	45, 189, 196
N^5−CHO−PDGA−H_4	±	+	+	*	45, 91
PTGA	−	+	*	+	45, 72, 107, 196
N^5−CHO−PTGA−H_4	−	−	−	*	45, 72, 91
PHGA	−	−	−	−	45, 107, 189, 196

[1]) Abbreviations: PA = pteroic acid, PGA = pteroylglutamic acid, PDGA = pteroyldiglutamic acid, PTGA = pteroyltriglutamic acid, PHGA = pteroylheptaglutamic acid, PGA−H_2 = dihydroPGA, PGA−H_4 = tetrahydroPGA.

[2]) + active, − inactive, * no data.

Both chicken pancreas and hog kidney conjugases have been widely used. Chicken pancreas enzyme seems to be more effective for different kinds of materials and is therefore usually preferred[23,90]. Some authors recommend the use of both types of conjugases for optimum release of folate[94,191].

h. Cobalamin

The responses of various micro-organisms to different cobalamins are summarized in Table 8[66]. *Ochromonas malhamensis* (ATCC 11532) is the most specific assay organism, since it responds only to clinically active forms of cobalamin. It has the disadvantage of requiring several days for growth[183]. *Euglena gracilis* (ATCC 12716) responds to the clinically active forms and also to some pseudoanalogues (but not to the noncobalamin analogues devoid of nucleotide); *Euglena* requires several days for growth[66,183]. *Lactobacillus leichmannii* (ATCC 7830) resembles *Euglena* in both sensitiveness and response to »true»- and some pseudo-cobalamins. It has the disadvantage of responding to deoxyribosides[66,183]. Desoxyribosides present in test material can be determined either by autoclaving it in alkaline medium, thereby destroying the cobalamin, and estimating the response of *L.leichmannii* to deoxyribosides[81] or by estimating deoxyribosides directly with

Table 8

Activity[1]) of cobalamins according to the base of the nucleotide[66].

Base of the nucleotide	*O. malhamensis*	*E. gracilis*	*E. coli*	*L. leichmannii*	Clinical activity
5,6-Dimethyl benzimidazole	100	100	100	100	+
Benzimidazole	36	+	200	200	+
5-Hydroxy benzimidazole	–	–	50	35	+
Adenine	0	100	100	50	–
2-Methyl adenine	0	60	100	40	–
Coenzyme forms:					
5,6-Dimethyl benzimidazole	100	–	100	–	–
Benzimidazole	50	–	100	–	–
Adenine	0	–	100	–	–

[1]) Taking the activity of the 5,6-dimethyl benzimidazole compound (vitamin B_{12}) as 100.

Lactobacillus thermoacidophilus (R 26), which does not respond to cobalamin[79]. *Eschericia coli* (ATCC 11105) responds to a wide variety of cobalamins including cobamide (Factor B) and methionine[66,183]. *E.coli* is usually suited to assay of pharmaceutical preparations only.

Most of the cobalamin activity in natural materials is present as coenzymes which are considerable less stable than cyanocobalamin. They are readily converted by light and heat to hydroxocobalamin which easily decomposes to inactive compounds. Therefore the extraction medium has to contain a reducing agent such as metabisulphite, ascorbic acid, thioglycollic acid or thiomalic acid and, in addition sodium cyanide in order to convert the labile hydroxocobalamin to the stable cyanocobalamin. Heating in an autoclave liberates the protein-bound cobalamins[66,183]. For animal tissues and milk digestion with papain and cyanide has been recommended[60]. Pseudocobalamins, which differ from »true» cobalamins in that the 5,6-dimethylbenzimidazole group is replaced by other groups such as adenine, do not possess animal vitamin activity as far as is known at present. It is, however, not likely that they give false response in food analyses, since they have been isolated from, faeces, sewage sludge, fermentation liquors and fermented fodders but have not been reported to occur in animal tissues and fluids[70,183].

III. OWN INVESTIGATIONS

A. Feeding of cows

O-cows were fed a ration containing the components shown in Table 9. The test cows received briquets, cellulose-rich paste and cellulose strips in different propositions according to appetite; thus the feed of one cow differed from that of another to some extent[212].

ULP-cows were fed daily rations of composition as presented in Table 10. The consumption of fodder units and urea per year is given in the table. The composition of the ration varied naturally during the lactation period depending on the milk production. The rations contained on the average 20%, 40% and 50% digestible true protein of the digestible crude protein. The numbers 20.1 and 20.2 refer to cow No. 20 after first and second calving respectively[212]. In their second lactations cows 20 and 22 were fed much more urea (550 g daily) during the period of highest milk production. The production was about 20% higher than that of the first lactation period.

The amounts of feed consumed annually by two O-cows (Jairu and Aino) and two ULP-cows (Lelo and Lila) and the corresponding annual milk yields are presented in Tables 11—14.

NorP-cows from farm 1 were on pasture or stall-feeding, when they received hay, silage, cereals and oat-straw. Cows from farm 2 on 18/4/67 were on stall-feeding, receiving hay, clover-hay silage, oats and molasses. On 2/10/67 cows of farms 2 and 3 were on pasture.

Table 9

Composition of the components of O-feed[212].

1. Composition of the briquettes, about 9 g each:

		1962—64 (%)	1965 (%)
a-Cellulose powder		9.5	9.9
Starch		57.0	52.9
Sucrose		20.9	23.1
Mineral salt mixture		8.2	8.9
Urea (94%) + ammonium salts (6%) (calc. as urea)		4.4	5.2
	Total	100.0	100.0
	Water content	15.0	15.0

2. Composition of the wet cellulose-rich paste:

		1962—63 (%)	1964— (%)
a-Cellulose powder		60.3	57.3
Starch		19.5	16.4
Sucrose		12.1	12.2
Mineral salt mixture		7.1	8.8
Urea		1.0	5.3
	Total	100	100
Mixed with water.	Water content	75	75

At need, more urea could be mixed with the paste or the sugar-free briquette powder, which was occasionally used.

3. Cellulose strips, 75% water, urea 4.0% + mineral salt mixture 3.0% in dry matter.
4. Vegetable oils, 50—130 g per cow per day.
5. Vitamins A (100 000 IU) and $D_2 + D_3$ (20 000 IU), from 1.1.65 also vitamin E.
6. Mineral salt mixture.

Table 10

Composition of the daily rations of ULP-cows. The consumption of fodder units and urea per year are also given[212].

Feed		Cow No.						
		20.1	20.2	21.1	22.1	23.1	24.1	25.1
Potatoes, kg	(d.m. 20%)	22.5	20.0	—	—	—	—	—
Sugar beet pulp, kg	(d.m. 90%)	3.5	7.0	4.7	4.7	—	—	—
Oats, kg	(d.m. 90%)	—	—	5.5	5.0	6.0	8.5	6.0
Barley, kg	(d.m. 90%)	—	—	1.5	2.0	4.0	2.0	1.5
Hemicellulose, kg	(d.m. 96%)	2.3	3.0	3.0	3.0	3.0	3.0	3.0
Silage, kg	(d.m. 20%)	—	—	—	—	—	—	16.0
Hay, kg	(d.m. 80%)	—	—	—	—	—	—	2.0
Straw, kg	(d.m. 80%)	1.5	0.3	1.0	1.0	2.0	2.0	—
O-fibre, kg	(d.m. 100%)	2.3[1])	1.0[2])	0.4[1])	—	—	—	—
Urea, g		440	560	440	440	350	370	340
Salt mixture, g		750	750	400	400	400	400	400
Feed units/day		10.2	12.9	12.8	12.7	12.3	12.4	12.3
Feed units/year		3365	~ 3750	3583	3875	3026	3549	3811
Urea, kg/year		136.0	155.0	123.1	129.3	93.9	95.1	89.0

[1]) O-fibre of low quality, digestibility about 50%.

[2]) O-fibre of high quality, digestibility 80%.

Table 11

Annual feed consumption and milk yield of O-cow Aino (third lactation period)[213].

Feed		Total amount fed, kg	Fodder units	Total N kg	Dig. urea N kg
Starch		1208		0.2	
Cellulose		1014	2410		
Sucrose		434			
Urea and ammonium salts		155.5		72.5	50.8
Vegetable oils		45.4	76		
Mineral mixture		250.3			
Vitamin A	mill. IU	33.6			
Vitamin D_2+D_3	mill. IU	6.6			
Vitamin E	g	130.3			
Total			2486	72.7	50.8
Annual milk yield				2316 kg	
Yield calculated on an energy basis				3080 »	
Yield calculated on a protein basis				3080 »	
Contents of total milk produced during the year:					
Dry matter				370.4 kg	16.0 %
Fat				143.6 »	6.2 »
Protein				98.7 »	4.26 »
Sugar				104.4 »	4.51 »

Table 12

Annual feed consumption and milk yield of O-cow Jairu (second lactation period)[213].

Feed		Total amount fed, kg	Fodder units	Total N kg	Dig. urea N kg
		1473		0.3	
Starch		740	2632		
Cellulose		672		79.0	54.6
Sucrose		167.9			
Urea and ammonium salts		37.3	63		
Vegetable oils		286.3			
Mineral mixture		35.9			
Vitamin A	mill. IU	7.2			
Vitamin D_2+D_3	mill. IU	138			
Vitamin E	g				
Total			2695	79.3	54.6
Annual milk yield				3484 kg	
Yield calculated on an energy basis				3832 »	
Yield calculated on a protein basis				4136 »	
Contents of total milk produced during the year:					
Dry matter				491.2 kg	14.1 %
Fat				159.4 »	4.6 »
Protein				132.2 »	3.80 »
Sugar				164.3 »	4.72 »

Table 13

Annual feed consumption and milk yield of ULP-cow Lelo (first lactation period)[213].

Feed		Total amount fed, kg	Fodder units	Total N kg	Dig. crude prot. N kg	True prot. N kg	Dig. true prot. N kg	Dig. urea N kg	Dig. »amide» N kg
		1431.7	1348	26.3	21.0	21.5	18.6		2.4
Oats		300.5	335	5.6	3.9	4.7	3.6		0.3
Barley		1440.0	1245	24.6	12.1	17.0	9.6		2.5
Sugar beet pulp, dry		130.0	43	0.6	0.2	0.5	0.2		0
Barley-straw		970.7	869						
Hemicellulose powder		62.8	52					42.3	
O-fiber		129.3		60.4	42.3				
Urea		120							
Fat from fodder fed		110.6							
Mineral mixture		36.4							
Vitamin A	mill. IU	7.3							
Vitamin D_2+D_3	mill. IU	142.7							
Vitamin E	g								
Total			3892	117.5	79.5	43.7	32.0	42.3	5.2
Annual milk yield						5653 kg			
Yield calculated on an energy basis						5509 »			
Yield calculated on a protein basis						5918 »			
Content of total milk produced during the year									
Dry matter							730 kg		12.9 %
Fat							212 »		3.7 »
Protein							190 »		3.35 »
Sugar							277 »		4.90 »

Table 14

Annual feed consumption and milk yield of ULP-cow Lila (first lactation period)[211].

Feed	Total amount fed, kg	Fodder units	Total N kg	Dig. crude prot. N, kg	True prot. N kg	Dig. true prot. N, kg	Dig. urea N kg	Dig. »amide» N kg
Potatoes (fresh weight)	6160	1176	15.4	13.8	6.7	6.1		7.7
Sugar beet pulp, dry	1171	900	19.8	9.8	13.8	7.9		1.9
Oat-straw	561	175	2.5	1.0	2.3	0.7		0.3
O-fiber	578	193						
Hemicellulose powder	847	770						
Urea	136		63.5	44.4			44.4	
Fat from fodder fed	35							
Fat from veget.oils	34	55						
Mineral mixture	212							
Vitamin A mill. IU	36.4							
Vitamin D_2+D_3 mill. IU	7.3							
Vitamin E g	202							
Total		3269	101.2	69.0	22.8	14.7	44.4	9.9
Annual milk yield						4777 kg		
Yield calculated on an energy basis						4873 »		
Yield calculated on a protein basis						4825 »		
Content of total milk produced during the year								
Dry matter						636 kg		13.3 %
Fat						198 »		4.14 »
Protein						155 »		3.24 »
Sugar						234 »		4.89 »

B. Collection of milk samples

Experimental milk from O-cows was collected at 2—4 weeks' intervals during the years 1963—68 and from ULP-cows during the years 1966—68.

Control milk from NorP-cows was collected from farm 1 at 2—4 weeks' intervals during the years 1963—65; later NorP-milk was studied only occasionally and the milk were received from farm 2 twice, 18/4/67 and 2/10/67 and from farm 3 once, 2/10/67.

The cooled samples of milks reached the laboratory within 4 hours, and if they could not be assayed immediately were stored at —20°C in a freezer.

C. Assay methods

1. Microbiological assays

Culture of the assay organisms. *L. casei* and *S. faecalis* were maintained in stab culture in a medium[156] containing 1% glucose, 1% sodium citrate, 0.5% tryptone and 20% (by volume) fresh yeast extract. (pH of the medium 6.8). To the stab culture medium 1.5% Bacto agar was added. Yeast extract was prepared by mixing 1 kg fresh yeast with 1 l water, incubating overnight at 37°C, heating in an autoclave to 100°C and centrifuging. The clear supernatant was sterilized 15 minutes at 0.8 Atm. When dried yeast extract (Difco[42]) was used in place of the fresh yeast extract the bacteria failed to grow.

L. fermenti, *L. leichmannii* and *L. plantarum* were maintained in stab culture in a medium[133] containing 1% tryptone, 1% beef extract, 2% glucose, 10% (by volume) fresh yeast extract, 0.2% dipotassium phosphate, 0.5% sodium acetate, 0.2% triammonium citrate, 0.02% magnesium sulphate and 0.05% manganese sulphate (pH of the medium 6.8). To the stab culture 1.5% Bacto agar was added.

N. sitophila was maintained on agar slants on Neurospora-culture agar[2] of the following composition: 0.5% dried yeast extract, 0.5% peptone, 4% maltose and 1.5% Bacto agar (pH of the medium 6.0—6.5). The unused Neurospora-agar was discarded after 4 weeks, since spore formation of *Neurospora* was very poor on old medium.

All stock cultures were transferred once a month to 3 new cultures, one of which served as the monthly stock culture; the two others were used for preparation of daily inocula. The bacteria were grown at 37°C overnight and *Neurospora* 5 days at room temperature. After growth they were stored in a refrigerator at about 3°C.

Preparation of inocula. The bacteria from the stab culture were used to inoculate the corresponding broth, which was incubated overnight at 37°C. The cells were harvested and washed 3 times with saline (0.9% sodium chloride solution) and the washed cells were suspended in 25 ml saline. The spores of *N. sitophila* were shaken directly off the agar slant into 25 ml saline. 1 drop (about 0.05 ml) of the inoculum was transferred aseptically to the sterilized assay tubes or flasks.

Assay media from Difco Laboratories were used, with the exception that for assays of nicotinic acid from the year 1966 onwards a synthetic medium recommended by Strohecker *et al.*[199] was adopted.

Assay design. Standard or unknown solutions, double-strength assay medium and distilled water were pipetted into test tubes (15×160 mm) as follows:

tube no.	ml standard or test solutions	ml distilled water	ml double-strength medium
0[1])	0.0	2.5	2.5
1, 2	0.0	2.5	2.5
3, 4	0.5	2.0	2.5
5, 6	1.0	1.5	2.5
7, 8	1.5	1.0	2.5
9, 10	2.0	0.5	2.5
11, 12	2.5	0.0	2.5

[1]) This tube was sterilized but not inoculated, and was used to set the colorimeter to zero.

For assays with *Neurospora* 50 ml Erlenmeyer flasks were used instead of test tubes.

The assay tubes or flasks were sterilized 5 minutes at 0.5 Atm. in an autoclave. This sterilization was adequate to prevent contamination in the bacterial assays, since the growth period was only 18 hours. Although *Neurospora* assays were incubated 5 days the sterilization was adequate for them too, due probably to the relatively simple medium (sucrose, biotin, thiamine and salts) and its low pH (4.5).

Incubation. Bacteria were incubated in the dark about 18 hours at 37°C in an air incubator. The *Neurospora* assays were incubated in the dark at room temperature (20—25°C) for 5 days.

Quantification. After incubation all assays were steamed to 100°C in order to stop growth. The turbidity of bacterial assays was measured at 600 mμ with a Klett-Summerson photoelectric colorimeter against the uninoculated tube 0. *Neurospora* mycelium was harvested, drained on filter paper, dried overnight at 60—70°C in an oven and the weight recorded to the nearest milligram.

Calculation of results. A standard curve was drawn by plotting the response of the test organism against increasing concentrations of the standard vitamin. The amount of vitamin in any given sample was then obtained by interpolating the standard curve according to the response of the sample tubes. The vitamin content of the sample tubes was multiplied by the dilution factor to obtain the vitamin content of the undiluted samples. The figures for the undiluted samples were averaged and the values, from several dilution levels, with deviation (deviation = difference between the mean and a single value) not greater than 10% of the mean, are reported.

Preparation of standard vitamin solutions. Thiamine hydrochloride, nicotinic acid, Ca-pantothenate, pyridoxal hydrochloride, biotin and cyanocobalamin were dissolved in distilled water, riboflavin and folic acid in 3% ethanol-water. To the folic acid solution a few drops of 2 N sodium hydroxide were added. From these stock solutions the working standard solutions were prepared. The same acid and/or enzyme reagent used to extract the vitamins from milk was added to the working standard solutions. All stock solutions were stored in a refrigerator at 3°C. Fresh stock solutions were prepared every other month.

2. Colorimetric estimation of riboflavin

25 ml milk and 7 ml trichloroacetic acid solution A (1000 g TCA and 150 ml water) were mixed together and the mixture allowed to stand overnight at room temperature in the dark. The extract was filtered and the precipitate washed several times with TCA solution B (28 ml solution A and 100 ml water). The filtrate was concentrated in a rotary evaporator to about 8 ml, poured into a calibrated tube and diluted to 10 ml. The greenish-yellow colour of riboflavin was measured with a Klett-Summerson photoelectric colorimeter set at 440 mμ. A standard curve was prepared from portions of pure riboflavin treated in the same manner as milk. With every group of estimations one sample of pure riboflavin was included to check the standard.

3. Extraction of B-vitamins from milk

In Table 15 the extraction methods used in this investigation are listed. In every procedure 10 ml milk and 10 ml water, acid or buffer were mixed together, heated if required and the pH adjusted to the enzymes' optimum. After hydrolysis the pH was adjusted to 4.5 when necessary, the digest was filtered, washed several times with water or buffer and the filtrate made up to 100 ml. From these extracts appropriate dilutions were made with distilled water for assay of individual vitamins.

The enzyme preparations used were as follows. Diastase (digestive power 1:250) and Papain (digestive power 1:350) from E. Merck A.G., Darmstadt, both of which were used without purification. Bacto Chicken pancreas from Difco Laboratories, Detroit, Michigan, USA was purified by the method of Iwai *et al.*[94]. Intestinal phosphatase and chicken liver enzyme were prepared in this laboratory by the method of Novelli *et al.*[155].

Table 15

Methods used for extraction of B-vitamins from O-, ULP- and NorP-milk.

Method no.	Water, acid or buffer added	Autoclave °C/min	pH	Enzymes added	Digest. °C/hours	Reference
1.	0.2 N HCl	121/10	4.5	Diastase	37/0.5	6 (Modified)
2.	0.4 M acetate buffer, ph 4.62	None	4.6	Diastase Papain	37/20	199
3.	0.2 N HCl	118/5	4.5	Diastase Papain	37/20	Modified from 1 and 2
4.	1 N HCl	121/60	4.5	None	None	199
5.	6 N H_2SO_4	121/60	4.5	None	None	50
6.	Water	118/10	4.5	None	None	160
7.	0.2 N »tris» buffer, ph 8.3	None	8.3	Intestinal phosphatase, chicken liver enzyme	37/20	155
8.	0.2 M phosphate buffer, pH 7.2 50 mg Na-ascorbate	118/15	7.2	Chicken pancreas enzyme	37/20	94
9.	0.1 N acetate buffer, pH 4.6 Na-ascorbate	60/15	4.6	Papain NaCN	60/1	176
10.	2 N KOH, pH 12	118/30	4.6	None	None	36
11.	Trichloroacetic acid	(Described separately in III B).				

Extraction method 1 was used from the beginning of the investigation until November/66 for the extraction of thiamine, riboflavin, nicotinic acid, pyridoxine and biotin. Method 2 was used for the extraction of thiamine and riboflavin from November/66 until the end of May/67. Method 3 was used from June/67 until the end of the investigation for the extraction of thiamine and riboflavin. Method 4 was used occasionally for the extraction of nicotinic acid. Method 5 was used for the extraction of nicotinic acid, pyridoxine and biotin from May/67 until the end of the investigation. Method 6 was used occasionally to assay the »free» forms of the vitamins. Method 7 was designed for the extraction of pantothenic acid and was used for the extraction of this vitamin only. Method 8 was used for the extraction of folate and sometimes for pantothenic acid also. Method 9 was used for the extraction of cobalamin. Method 10 was a control treatment for cobalamin assay to check the absence of deoxyribosides. For riboflavin a colorimetric method (no. 11) was developed and used in addition to the microbiological method.

Table 16 lists the assay conditions for each vitamin.

Table 16

B-vitamin assay conditions (other details in text).

Vitamin	Assay organism	Assay medium (Difco)	Method of extraction (Table 15)	Milk added µl/ml	Concentration of standard vitamin mµg/ml
Thiamine	*L. fermenti* ATCC 9338	326	1, 2, 3, 6	4–40	1.5–15
Riboflavin	*L. casei* ATCC 7469	325	1, 2, 3	2–20	5–50
Nicotinic acid	*L. plantarum* ATCC 8014	322	1, 4, 5	3–30	5–50
Pantothenic acid	*L. plantarum* ATCC 8014	604	6, 7, 8	0.5–5	4–40
Pyridoxine	*N. sitophila* ATCC 9276	324	1, 5, 6	5–50	2–20
Biotin	*L.plantarum* ATCC 8014	419	1, 5, 6	0.5–15	25–250[1])
Folic acid	*S. faecalis* ATCC 8043	318	6, 8	6–60	0.1–2
Folic acid	*L. casei* ATCC 7469	822	6, 8	1.5–15	0.4–4
Cobalamin	*L.l eichmannii* ATCC 7830	457	6, 9, 10	5–50	25–250[1])

[1]) µµg/ml.

4. Explanation of terms used

For practical reasons, one basic name was generally used for every vitamin although it was known that 1. several forms of the vitamin could be present in milk, as in the case of folate, or 2. the vitamin in question occurs in a special form in milk. For example nicotinic acid is in the form of nicotinamide in milk.

»The concentration of the vitamin in milk» means the response of the assay organism under the test conditions to all vitamin activity present in the sample, expressed in terms of concentration of the standard vitamin. For instance, »the concentration of cobalamin in milk» means the response of *L. leichmannii* to all B_{12} activity (including all clinically active forms, pseudocobalamins and deoxyribosides but not noncobalamin analogues)[183] in milk under the experimental conditions, expressed as cyanocobalamin.

5. Comments on assay methods

Vitamins were extracted from milk by methods which in some cases were quite rigorous. Though unphysiological, these methods were chosen because they are generally accepted for vitamin analysis, and the extracts obtained were well suited for microbiological assay techniques. In addition, it should be noted that for the present animal vitamin assays are the only ones for which no extraction procedure is necessary.

It is not known whether microbiological techniques reveal the »true» vitamin activity useful for man. However, when the aim of this investigation was to obtain information about the vitamin concentration in the milks of cows on experimental and conventional feeds, it was reasonable to assume that microbiological methods would give results permitting direct comparison. Besides, when biotin, folate and cobalamin had to be estimated microbiologically due to their low concentration in milk, microbiological methods, being relatively rapid and reliable, were chosen for the assay of all B-vitamins.

In addition, the coenzyme forms of vitamins in natural materials are very complicated and knowledge of them is still so scanty that, if animal assays are excluded, it is extremely difficult to judge which of the methods now available for vitamin analysis can be considered the »best».

D. Results and discussion

1. Evaluation of the methods

a. Extraction

The extraction methods used are summarized in Table 15. The enzyme preparations were tested and found to be free of the vitamin under assay in each case.

Thiamine. Extraction with methods 1 and 3 gave the same results: for instance, both methods gave a mean concentration of 46 μg of thiamine/100 ml O-milk. The difference between these methods was that only diastase was used in method 1 whereas both diastase and papain were used in method 3. When the heat treatment was omitted and the two enzymes were used together (method 2) the mean concentration of thiamine in O-milk found was 18 μg/100 ml. Sarett *et al.*[175] reported that treatment with heat and

both phosphorolytic and proteolytic enzymes is necessary to obtain reliable results in thiamine assays. According to the results presented here, treatment with heat and phosphorolytic enzyme (diastase) but not with proteolytic enzyme (papain) is necessary to extract thiamine in free form from milk.

Riboflavin was extracted with the same methods as thiamine. With method 1 the concentration of riboflavin in O-milk was higher (301 μg/100 ml) than with the other two methods (193 μg/100 ml with method 2 and 199 μg/100 ml with method 3). The concentration of riboflavin in NorP-milk was 201 μg/100 ml with method 1, 102 μg/100 ml with method 2 and 171 μg/100 ml with method 3. The colorimetric method (No. 11) gave a value of 164 μg/100 ml. Method 2 was considered to be unreliable, since the figure obtained for NorP-milk by this method was considerably lower than those obtained with the other three methods (1,3 and 11).

The high level of riboflavin in O-milk obtained with method 1 was not due to the superiority of this method but merely to higher levels of riboflavin in the milk of the earlier O-cows. The concentration of riboflavin in the milk of individual cows found by the colorimetric method show that in the milk of the earlier O-cows (Aino and Eiru) the content of riboflavin was about 300 μg/100 ml whereas in the milk of the later O-cows (Jairu, Metta and Nairu) it was a little below 200 μg/100 ml (Table 21). The higher figures obtained at the beginning of the investigation were thus a reflection of individual characteristics.

The colorimetric estimation of riboflavin was developed because the internal error of the microbiological assays tended to be greater than ±10% of the mean, with either upward or downward »drift» (»drift» = increasing or decreasing calculated vitamin concentration as the amount of sample solution per tube was increased or decreased).

The values obtained with the colorimetric method were slightly lower than those obtained with the microbiological methods.

Nicotinic acid could be extracted either by heating in dilute mineral acid with subsequent digestion with diastase (method 1) or by drastic acid hydrolysis without enzyme treatment (method 5). The concentration of nicotinic acid in O-milk was 200 μg/100 ml with the dilute acid/diastase method and 175 μg/100 ml with the vigorous extraction method. Treatment with dilute acid without subsequent diastase digestion (method 4) gave a nicotinic acid value of 95 μg/100 ml O-milk. In one test a sample of NorP-milk was extracted according to methods 1 and 4 and the concentrations of nicotinic acid found were 179 μg and 96 μg/100 ml respectively. It has been claimed that mere heating with water or with dilute acid will

liberate nicotinic acid from milk and other material[116,139]. The results presented here indicate that vigorous acid extraction or enzyme treatment after heating with dilute acid is necessary for extraction of nicotinic acid from milk.

Pantothenic acid was generally extracted by digestion with intestinal phosphatase and chicken liver enzyme[155]. However, it was observed later that extraction with purified chicken pancreas enzyme (method 8) was as effective as the method of Novelli *et al.*[155]. The concentration of pantothenic acid in one O-milk with both methods was about 1000 μg/100 ml. Chicken pancreas digestion is now preferred for the extraction of pantothenic acid from milk, since folate, for which the method was originally developed[94,122], can be estimated on the same extract, and in addition the use of chicken pancreas enzyme is simpler than that of intestinal phosphatase and chicken liver enzyme.

According to earlier reports[8,75,78,123,192] pantothenic acid could be extracted from milk by heating in water. However, the finding that about 25% of pantothenic acid in milk is in a bound form[70] and the demonstration of considerable amounts of CoA in milk[74], indicate the necessity of enzymatic digestion. Gregory[61] extracted pantothenic acid from milk by intestinal phosphatese and chicken liver enzyme; the values obtained were slightly higher than those obtained with untreated milk.

Pyridoxine. Extraction of pyridoxine with dilute acid followed by diastase digestion (method 1) gave about 50 μg pyridoxine/100 ml O-milk. When drastic acid extraction (method 5) was used, the concentration of pyridoxine fell to about 30 μg/100 ml. The milk of one O-cow (Jairu) was assayed with both methods; the mean concentration of pyridoxine was 46 μg/100 ml with method 1 and 35 μg/100 ml with method 5. Evidently the yield of pyridoxine is a reflection of the method used.

The reason for the difference may be that: 1. Heating with 6 N sulphuric acid for 1 hour at 121°C either destroys part of the pyridoxine in milk or rebinds the liberated pyridoxine to proteins, as suggested by Storvick *et al.*[198]. 2. Since the relative response figures of *N. sitophila* in the presence of excess thiamine, which is added to the medium to increase the sensitivity and precision of the method[69], are 1.0, 1.1 and 0.3 respectively for pyridoxol, pyridoxal and pyridoxamine[77], the pyridoxine results will be erroneous if pyridoxamine forms a considerable part of the total pyridoxine activity of milk. The relative responses obtained for *N. sitophila* in this investigation were, on a molar basis: pyridoxol 1.0, pyridoxal 1.0, pyridoxamine 0.25 and pyridoxal phosphate 0.50. 3. In fresh milk pyridoxal accounts for about 80% of the total pyridoxine activity, the rest being pyridoxamine[64]. How-

ever, the amount of pyridoxamine in milk extract prepared by method 5 may have been considerably higher than that indicated by Gregory's figures[64], since during the heating a considerable part of the pyridoxal was certainly converted to pyridoxamine by interaction with amino acids[189].

Taking the possible effect of vigorous acid hydrolysis and the use of excess thiamine in the assay medium into consideration, the reason for the lower levels of pyridoxine obtained with method 5 may be rebinding of pyridoxine to proteins and the presence of considerable amounts of pyridoxamine in the sample. The more vigorous hydrolysis was preferred, since the method is precise and the results were considered to be inter-comparable. Reducing the heating period to 15—30 minutes could be advantageous, but this was not investigated.

Biotin. Extraction of biotin from O-milk with dilute acid and diastase (method 1) or with concentrated acid (method 5) gave different results, namely 3.8 μg and 8.5 μg/100 ml respectively. Method 1 was used until the end of 1966 and method 5 thereafter. The level of biotin in one O-milk was estimated during both periods and consequently with both methods. Before the end of 1966 the concentration was 3.9 μg/100 ml (method 1) and after 1966 it was 8.8 μg/100 ml (method 5). These results indicate a difference in the efficacy of the extraction methods. However, in NorP-milk the concentration of biotin was 2.9 μg/100 ml (method 1) and 1.6 μg/100 ml (method 5). The situation is thus the reverse of that in O-milk. In addition, when 3 samples of O-milk were treated with water at 118°C for 10 minutes (method 6) and 3 with 6 N sulphuric acid at 121°C for 1 hour (method 5) the results obtained were the same, namely 11.2 μg/100 ml (Table 23). In the light of these results there was a true difference in biotin levels in O-milk before and after the end of 1966. The case is discussed further in Section D.2.a.

For extraction of biotin from milk many authors have suggested autoclaving the sample with water, since their assay results were not changed by treatment with acid or enzyme[78,123,192,225]. The results obtained here with O-milk and methods 5 and 6 support this view. However, the biotin figures obtained with method 5 for ULP-milk were about twice those obtained with method 6 (Table 23). Extraction 5 was better suited for microbiological estimation and was therefore used.

Folic acid extraction by heating with water (method 6) gave 2.9 μg and by heating with buffer followed by chicken pancreas digestion (method 8) 2.2 μg/100 ml O-milk, when *S. faecalis* was used as the assay organism. When folate was extracted from NorP-milk (pooled dairy milk) by the

same methods and assayed with *L. casei* the concentrations found were about 10 times higher, namely 25 and 32 μg/100 ml respectively for water and enzyme extraction. It was concluded that method 8/*L. casei* assay is superior for the determination of milk folate, which conclusion is supported by the finding of Kazenco *et al.*[106] that folate which is easily destroyed by acid or alkali is liberated by chicken pancreas enzyme without destruction and by the fact that *L.casei* responds to N^5-methylfolates[121].

Cobalamin was extracted with papain digestion in the presence of ascorbic acid and sodium cyanide (method 9). Addition of ascorbic acid or some other reducing agent is necessary to protect the labile forms of cobalamin.[176]

Since *L. leichmannii* responds to deoxyribosides, if present in high concentration[36], their presence in the milk samples was tested by destroying the cobalamin with alkali (method 10) and estimating any remaining activity with *L.leichmannii*. The extracts were found to be inactive.

b. Assay

To test the between-assay variation, the B-vitamin content of single milk samples from different cows was assayed several times. The deviations of the values obtained (Table 17) were within 10% of the means except those of riboflavin in the milk of cow 3 and those of biotin in the milk of cow 2. The reason for the large error in these two cases is not known. In many instances the deviation of the values obtained from the same sample were 5% of the mean or even less. To test the between-extraction variation, the content of pantothenic acid, biotin and cobalamin was determined in several extracts made from single milks of different cows. The deviation obtained with each sample were within 6% of the mean (Table 18). The data presented show that with the two exceptions (Table 17) the reliability of the methods used is acceptable for biological methods.

The reproducibility of the colorimetric riboflavin assay is shown in Table 19. The deviations were within 9% of the mean.

The numbering of the cows in these Tables (17—19) is arbitrary; thus the mean values in any one column are not necessarily for the milk of the same cow.

Table 17

Between-assay variation of B-vitamin values (μg/100 ml) in single samples of milk.

Vitamin	Assay no.	Cow				
		1	2	3	4	5
Thiamine	1	35	39	40	45	41
	2	39	43	47	44	40
	3					42
	4					41
	Mean	37	41	44	45	41
Riboflavin	1	208	104	170	192	203
	2	201	110	116	188	184
	3					204
	4					188
	Mean	205	107	(143)	190	195
Nicotinic acid	1	197	153	136	176	156
	2	167	157	153	200	178
	3	174	137	149	188	171
	Mean	179	149	146	188	168
Pantothenic acid	1	994	424	1002		
	2	984	440	1160		
	3			1112		
	4			1102		
	Mean	989	432	1094		
Pyridoxine	1	22				
	2	24				
	Mean	23				
Biotin	1	1.0	1.4	12.0	7.9	
	2	0.9	1.2	11.5	8.0	
	Mean	1.0	(1.3)	11.8	8.0	
Folic acid (*L. casei*)	1	28	47			
	2	27	42			
	Mean	28	45			
Cobalamin[1])	1	495	314	600		
	2	543	338	586		
	Mean	519	326	593		

[1]) mμg/100 ml.

Table 18

Between-extraction variation of pantothenic acid, biotin and cobalamin values (μg/100 ml) in single samples of milk.

Vitamin	Extraction No.	Cow	
		1	2
Pantothenic acid	1	996	1200
	2	1020	1336
	Mean	1008	1268
Biotin	1	0.98	
	2	0.98	
	3	0.90	
	Mean	0.95	
Cobalamin[1])	1	284	195
	2	274	183
	Mean	279	189

[1]) mμg/100 ml

Table 19

Variation of riboflavin values (μg/100 ml) determined colorimetrically in single samples of milk.

Assay No.	Cow			
	1	2	3	4
1	132	148	182	186
2	143	144	186	182
3	156	148		
4		146		
5		136		
Mean	144	144	184	184

c. Erratic behaviour of assay organisms

Difficulties, the cause of which could not be traced, occurred in the assay of every vitamin from time to time.

1. All bacteria grew well in the appropriate stab culture medium but occasionally failed to grow for periods of weeks in the corresponding liquid medium, even though fresh medium was prepared. The enrichment of the

liquid medium with some nutrient-rich extract, for example the addition of a tomato extract to the *L. leichmannii* medium, did not always result in growth. When the inoculation was repeated after some months the same organism grew perfectly well.

2. Sometimes the organism grew well in both solid and liquid media but failed to grow in the assay medium.

3. In contrast, the phenomenon of unrestricted growth of the organism in every assay tube, including those with no essential vitamin, was observed. The uninoculated tube for the colorimeter blank was the only one with no growth. Microscopical examination showed that there was no contamination with other organisms. The assay medium had been taken from the same flask that had furnished, at other times, media which gave satisfactory assays. Again, after some months the behaviour of the assay organism returned to normal.

4. In some cases improvement could be achieved by modification of the assay medium. For example, in cobalamin assays the rather uneven response of *L. leichmannii* to cobalamin could be corrected by adding 0.1% ascorbate to the Difco assay medium.

5. The growth of *N. sitophila* in pyridoxine assays was very irregular at the beginning of the investigation. Addition of thiamine (5 mg/100 ml medium) as suggested by Harris[69] improved the variation of the assay results to within 10% of the mean. Unfortunately, the addition of thiamine changed the response of *Neurospora* to different forms of pyridoxine[77].

6. When folate was assayed with *L. casei* using different synthetic media recommended in the literature[14,45] the bacteria often failed to grow or grew equally well in all assay tubes, including those with no added folate. When Difco medium no. 0822 was compounded according to directions in »Microbiological assay of vitamins and amino acids»[42] *L. casei* failed to grow. Not until the same medium (0822) was ordered ready-made from Difco Laboratories did the assay succeed. This failure was supposed to be due to lack of impurities in the chemicals used in the laboratory preparation of the medium. Apparently, some necessary growthfactor was present in the commercial Difco product.

2. B-vitamin content of milk

a. B-vitamin concentration in O-, ULP- and NorP-milk

In Table 20 the mean concentration of B-vitamins in O-, ULP- and NorP-milk during the years 1963—68 are presented. Apparently, the feed had no significant effect on the concentration of thiamine, nicotinic acid,

Table 20

B-vitamin concentration (μg/100 ml, with standard errors) in the milk of O-, ULP- and NorP-cows during the years 1963—68. (Number of samples assayed in parentheses).

Vitamin, Extraction method no(s)[1])	O-milk	ULP-milk	NorP-milk
Thiamine	46.0±1.4	46.2±2.5	41.8±1.5
1, 3	(42)	(21)	(22)
Riboflavin	284**[2])±15	206±12	201±21
1, 3	(31)	(3)	(5)
Riboflavin	223***±12	173±7.3	164±7.9
11	(32)	(41)	(13)
Nicotinic acid	191±5.9	174±12	173±5.8
1, 5	(55)	(20)	(19)
Pantothenic	982***±36	349±34	443±40
acid, 7, 8	(47)	(18)	(18)
Pyridoxine	52.2±3.1	—	50.2±4.1
1	(19)		(12)
Pyridoxine	32.7±1.1	30.4±1.4	—
5	(9)	(9)	
Biotin	3.83±0.34	—	2.92±0.49
1	(19)		(9)
Biotin	8.45***±0.72	1.46±0.15	1.60±0.13
5, 6	(22)	(42)	(28)
Folic acid	2.57±0.24	2.06±0.28	3.18±0.19[3])
(*S. faecalis*), 6, 8	(11)	(5)	
Folic acid	36.4±2.7	30.5±1.7	32.0±12
(*L. casei*), 8	(16)	(22)	(2)
Cobalamin[4])	394±27	1295***±88	621*±80

[1]) Extraction methods numbered according to Table 15.

[2]) Levels of significans are indicated as follows: * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

[3]) Values for 1963 only. (Syvänen, R., 1963).

[4]) mμg/100 ml.

pyridoxine or folate, whereas it affected the concentration of riboflavin, pantothenic acid, biotin and cobalamin.

Thiamine. The mean concentration of thiamine, namely 46, 46 and 42 μg/100 ml respectively for O-, ULP- and NorP-milk agree relatively well with the value of 38 μg/100 ml (range 26—46) obtained with fluorometric or chemical methods[29,30,68,85].

Riboflavin. The mean levels of riboflavin were 284 and 223 μg/100 ml O-milk, 206 and 173 μg/100 ml ULP-milk and 201 and 164 μg/100 ml NorP-milk with the microbiological and colorimetric method respectively. The concentration was higher in O-milk than in the other two milks, the

difference being significant ($P < 0.01$) with the microbiological method and highly significant ($P < 0.001$) with the colorimetric method.

There was no statistical difference between ULP- and NorP-milk with regard to riboflavin concentration found with either method, however, the riboflavin values for all milks were somewhat lower when the colorimetric method was used.

The levels of riboflavin in milk obtained with the fluorometric method range from 86 to 300 μg/100 ml[30,42,145] and with the *L.casei* assay from 122 to 250 μg/100 ml[40,78,184]. The concentration in O-milk was somewhat higher than most of the values reported in the literature. The riboflavin content of ULP- and NorP-milks agreed relatively well with the values reported elsewhere.

Nicotinic acid. The mean concentration of nicotinic acid, 191, 174 and 173 μg/100 ml respectively for O-, ULP- and NorP-milk, were higher than the values (56—114 μg/100 ml) obtained with *L. plantarum* assays[30,75,78,123,192]. When nicotinic acid was extracted with dilute mineral acid without enzymatic digestion (method 4) the value obtained, 95 μg/100 ml O-milk, was nearer to the literature values.

Pantothenic acid. The difference in pantothenic acid concentration between O- and the other two milks was highly significant, the mean concentrations being 982 μg/100 ml O-milk, 349 μg/100 ml ULP-milk and 443 μg/100 ml NorP-milk. There was no statistical difference between the ULP- and NorP-milk values. The concentration of pantothenic acid in ULP- and NorP-milks was on about the same level as values obtained with *L. plantarum* assays, namely 190—420 μg/100 ml[30,75,78,123,192]; the concentration in O-milk was considerably higher.

Pyridoxine. There was no significant difference in pyridoxine concentration between O- and NorP-milks (52.2 and 50.2 μg/100 ml respectively) during the years 1963—65 or between O- and ULP-milks (32.7 and 30.4 μg/100 ml respectively) after 1965. However, the concentration of pyridoxine showed a tendency to decline. This decline is thought to originate from the change of the method in 1966 as discussed in Section D.1.a. The concentration of pyridoxine in milk reported by other investigators varies: 51 μg/100 ml with *S. cerevisiae* assays[178], from 9 to 60 μg/100 ml with *S. carlsbergensis* assays[7,30,63,77,228], 32 μg/100 ml with *L. casei* assays[170] and from 34 to 58 μg/100 ml with *N. sitophila* assays[77,133].

Biotin. From 1963 until 1965 there was no significant difference in the concentrations of biotin in O- and NorP-milk, which were 3.83 and 2.92 μg/100 ml respectively. However, a marked increase of the biotin level in O-milk occurred during the later years of the investigation, the level then being significantly higher ($P < 0.001$) than the levels in the other two milks. The mean concentrations of biotin were 8.45 μg/100 ml O-milk, 1.46 μg/100 ml

ULP-milk and 1.60 μg/100 ml NorP-milk. There was no statistical difference between the last two figures. The biotin values of ULP- and NorP-milk agree quite well with those obtained with *L. plantarum* assays (1.1—3.7 μg/100 ml[30,123]); the figures obtained with *L. casei* assays were slightly higher (1.3—6.2 μg/100 ml)[63,78,177,192].

As noted in Section D. 1.a. the difference between the earlier and later O-milk samples with regard to biotin concentration was thought to be due to a rise in the concentration rather than to any shortcoming in the extraction procedure. Such a rise is difficult to explain. However, there were two marked changes in the feeding of the cows during this period. 1. At the end of 1965 the amount of urea in the feed was increased by about 25%. 2. In January/66 a tocopherol supplement (about 400 mg/day/cow) was added to the feed of both O-and ULP-cows. After these two changes a slow decline in milk fat content was observed. The mean concentration of biotin (17 estimations) in O-milk before 1966 was 3.8 μg/100 ml (range 1.8—8.0) and the mean fat content on the days when the milk samples were taken was 5.7% (range 4.0—7.1). After 1966 the corresponding means (25 estimations) were 8.5 μg/100 ml (range 4.9—16.5) and 3.7% (range 3.1—5.1) respectivey for biotin and fat. The negative correlation between fat and biotin concentrations in O-milk was not always found in individual samples although it was clear in the means of all samples.

In ULP-milk the mean fat content was 3.9% (range 1.7—6.0) and the biotin concentration 1.5 μg/100 ml (range 0.3—4.8), both of which can be considered normal values.

It is possible that the opposed changes in fat and biotin contents in O-milk were the result of some permanent change in rumen population due to these alterations in feeding. It is well known that biotin acts as a coenzyme in fatty acid synthesis[53,215,216,217]. Probably later the lower rate of production of fat by the O-cows entailed the usage of less biotin and therefore there was more available for secretion into milk.

Folic acid. When folate activity was estimated with *S. faecalis* it was slightly higher (3.18 μg/100 ml) in NorP-milk than in O- or ULP-milks (2.57 and 2.06 μg/100 ml respectively). However, none of the differences between the 3 figures was significant. When folate was estimated with *L. casei* the level was about 10 times higher in all 3 milks. The concentration was slightly higher in O-milk (36.4 μg/100 ml) than in ULP- and NorP-milk (30.5 and 32.0 μg/100 ml respectively) but the difference was not significant.

The difference in folate activity obtained with *S. faecalis* and *L. casei* assays indicates that folate activity in milk is due to N^5-methylfolates, the principal forms of folate in blood and liver[72,153], since only *L. casei* responds to these forms[148,149,173].

The concentration of folate obtained with *S. faecalis* assays ranges from 0.06 to 0.60 μg/100 ml[36,103] and with *L. casei* assays from 4.2 to 12.4 μg/100 ml[104,148,200]. The folate values obtained with both organisms in this investigation were higher than these literature values. The fact that in this work ascorbate was added to the extraction and assay media to protect the labile folates[54] explains the higher values obtained with *S. faecalis*. The higher values obtained with *L. casei* cannot be due to the use of ascorbate, since other investigators also have used it. Probably the concentration of folate in these milks was higher than reported elsewhere.

The *cobalamin* concentration was highest in ULP-milk (1295 mμg/100 ml); the difference between this figure and the O- and NorP-milk figures was highly significant. The concentration of cobalamin in NorP-milk (621 mμg/100 ml) was higher than that in O-milk (394 mμg/100 ml); the difference was almost significant.

The concentration of cobalamin in ULP-milk was very high; some authors have reported comparable values, namely from 130 to 1400 mμg/100 ml with *E. gracilis*[157] and from 596 to 1200 mμg/100 ml with *L. leichmannii*[109]. Other values obtained with *L. leichmannii* range from 260 to 760 mμg/100 ml[3,129] and with *O. malhamensis* it was 320 mμg/100 ml[30].

b. B-vitamins in the milk of individual cows

The mean concentration of B-vitamins in the milk of individual cows and in pooled milk is presented in Table 21. There were great differences within each group, the variation in the pooled NorP-milk figures being not so marked. The variation of pyridoxine was slightly less than that of the other vitamins.

Considerable variation between cows with regard to the concentration of different vitamins in their milk has been reported, the variation of biotin, cobalamin and folate being greatest[52,63,103,104,123].

c. Short-term variation in the levels of nicotinic acid, pyridoxine and biotin in O- and ULP-milk

Table 22 shows the variations in the concentrations of biotin, nicotinic acid and pyridoxine in the test milks between successive milkings. The fat and protein contents of the same milk samples were estimated also in order to compare the variation of other milk constituents with that of the 3 vitamins.

Table 21

The mean concentration (μg/100 ml) of B-vitamins in the milk of single cows and in pooled NorP-milk.

Vitamin	O-milk						ULP-milk						Pooled NorP-milk			
	Cow: Aino	Eiru	Jairu	Metta	Nairu	Oona	Euru	Kairu	Kelo	Kila	Lelo	Lila	1	2	3	4
Thiamine	49	48	42	39	55	—	42	41	41	63	57	45	42	—	43	41
Riboflavin	308	293	185	207	161	—	138	155	141	161	203	213	—	164	—	—
Nicotinic acid	208	196	166	233	125	—	118	131	188	140	207	234	172	—	179	—
Pantothenic acid	993	1063	1041	656	897	960	226	333	210	397	411	382	493	—	312	—
Pyridoxine	—	—	35	—	30	—	27	34	36	—	—	30	—	—	—	—
Biotin	—	—	8.8	—	6.4	11.2	0.6	1.9	1.6	1.9	1.1	1.5	—	1.5	1.1	2.3
Folic acid (*L. casei*)	—	—	24	31	38	40	33	27	29	42	28	30	32	—	—	—
Cobalamin[1])	480	505	324	324	464	376	960	1272	1018	1969	1151	1354	586	532	—	710

[1]) mμg/100 ml.

Table 22

Variations in the contents of nicotinic acid, pyridoxine, biotin, fat and protein in O- and ULP-milk between successive milkings.

Cow	Date/time of milking	Nicotinic acid	Pyridoxine	Biotin	Fat %	Protein %	Milk yield kg
		μg/100 ml					
	1968						
Jairu	19/1 pm.	191	33	6.0	3.19	4.17	2.30
(O)	20/1 am.	206	—	5.6	3.21	4.09	3.25
	20/1 pm.	208	34	6.0	3.29	4.11	2.27
	21/1 am.	228	—	5.2	3.30	4.04	3.27
	21/1 pm.	195	35	5.3	3.40	4.11	2.25
	22/1 am.	223	—	4.4	3.40	4.11	3.22
	Mean	209	34	5.4	3.28	4.10	2.76
Nairu	19/1 pm.	226	27	5.5	3.50	3.84	1.28
(O)	20/1 am.	164	—	5.1	3.10	3.87	1.67
	20/1 pm.	247	30	6.5	3.28	3.65	1.29
	21/1 am.	165	—	6.0	3.06	3.86	1.60
	21/1 pm.	223	29	8.5	3.50	3.91	1.24
	22/1 am.	200	—	9.1	3.50	3.91	1.55
	Mean	204	29	6.8	3.29	3.83	1.44
Lila	19/1 pm.	243	26	1.3	3.24	3.70	6.77
(ULP)	20/1 am.	—	—	1.6	3.88	3.67	9.00
	20/1 pm.	242	28	2 1	3.99	3.68	6.60
	21/1 am.	—	—	1.7	3.42	3.74	8.80
	21/1 pm.	241	26	1.1	3.40	3.65	6.82
	22/1 am.	—	—	0.9	3.40	3.65	8.90
	Mean	242	27	1.5	3.62	3.69	7.82

The variation of nicotinic acid and biotin in one series of O-milks (Nairu) and the variation of biotin in the ULP-milk (Lila) were great. In the other O-milk (Jairu) the deviation of nicotinic acid was less than 10% of the mean and that of biotin less than 12% of the mean, except on 22/1/68 when the deviation was 18% of the mean. The variation of nicotinic acid in ULP-milk and of pyridoxine, fat and protein in all milks was small. The changes of biotin and nicotinic acid levels seem to be quite unrelated.

d. »Free» and »total» B-vitamins in milk

In Table 23 the concentrations of »free» and »total» vitamins in O- and ULP-milk samples are given. The »free» vitamin means that portion of the vitamin which can be extracted from milk by heating with water in an autoclave (method 6). Obviously, some of this »free» vitamin is originally loosely bound to protein and is liberated by heating. The »total» vitamin means the amount extracted with acid and/or enzymes as indicated in

Table 23

The concentrations (μg/100 ml) of »free» and »total» B-vitamins in milk. The nicotinic acid values are means of several estimations; the other values are single estimations.

Vitamin	Milk no.	O-milk		Milk no.	ULP-milk	
		»Free» vitamin	»Total» vitamin		»Free» vitamin	»Total» vitamin
Thiamine	1	23	38	1	35	42
Nicotinic acid		95	174		115	191
Pantothenic acid	1	930	996	1	340	424
	2	935	1096	2	341	481
				3	400	552
	Mean	933	1046		360	485
Pyridoxine	1	21	35	1	27	26
Biotin	1	9.7	9.3	1	1.90	0.95
	2	12.8	11.4	2	0.43	0.65
	3	11.5	12.4	3	1.60	3.41
				4	2.00	4.82
				5	1.30	3.43
				6	0.50	0.70
	Mean	11.2	11.2		1.29	2.33
Folic acid[1])	1	35	44			
	2	15	20			
	Mean	25	32			
Cobalamin[2])	1	320	300	1	620	700
	2	300	460	2	760	860
	Mean	310	380		690	780

[1]) Dairy milk, *L. casei* assay.

[2]) mμg/100 ml.

Table 15. In the case of nicotinic acid the »free» vitamin was extracted by dilute acid (method 4) and the »total» vitamin by 6 N acid (method 5).

As far as any conclusion can be drawn from only a few microbiological assays, the amounts of »total» thiamine, pantothenic acid, nicotinic acid and folate were somewhat higher than the amounts of the »free» vitamins in both O- and ULP-milk. The amount of »total» cobalamin was only slightly higher than that of the »free» vitamin in both milks. The amount of »free» pyridoxine in O-milk and »free» biotin in ULP-milk was smaller than the amount of the »total» vitamins.

The amounts of »free» and »total» vitamins were determined to find out whether special extraction methods were necessary. If a higher value can be held as a criterion of better extraction, then it is preferably to use special extraction methods.

e. B-vitamin concentration in colostrum

In Table 24 the concentration of B-vitamins in the colostrum of the O- and ULP-cows is presented. Taking into consideration the relatively large variations of B-vitamin concentrations in the milk of individual cows and the limited number of estimations of colostrum vitamins, the values obtained are only an indication of the B-vitamin status of O- and ULP-colostrum. However, comparison with values obtained for the corresponding milk samples shows differences between colostrum and milk with regard to thiamine, folate and cobalamin, their content in colostrum being clearly higher than in milk. The concentration of pantothenic acid in colostrum was lower than in milk. Differences between colostrum and milk with regard to riboflavin, nicotinic acid, pyridoxine or biotin concentrations were not shown clearly.

Table 24

Concentration (μg/100 ml) of B-vitamins in the colostrum of O- and ULP-cows. The corresponding milk values are given in parentheses.

Vitamin	O-cows	ULP-cows
Thiamine	79 (46)	90 (46)
Riboflavin	194 (223)	226 (173)
Nicotinic acid	204 (191)	244 (174)
Pantothenic acid	613 (982)	132 (349)
Pyridoxine	34 (33)	—
Biotin	10.00 (8.45)	0.45 (1.46)
Folic acid (*L. casei*)	102 (36)	—
Cobalamin[1])	1900 (394)	2800 (1295)

[1]) mμg/100 ml.

IV. GENERAL CONSIDERATIONS

The purpose of this investigation was to elucidate to what extent the concentration of B-vitamins in the milk of the cow depends on an external source of these vitamins or whether the rumen population is able to synthesize them in necessary amounts. According to the literature this question is still partly unsolved. The remarkable discovery in this laboratory that a considerable production of milk is possible with a purified protein-free ration, urea and ammonium salts being the sole source of nitrogen, offered an opportunity to study this problem.

Comparison of the B-vitamin concentration of milk (O-milk) produced on such a ration with that of milk (NorP-milk) produced on a conventional protein-rich ration showed that the concentration of several of the vitamins in both milks was on about the same level. However, in O-milk the concentration of pantothenic acid, riboflavin and biotin was markedly higher than in NorP-milk.

In milk produced with urea-rich/low-protein rations (ULP-rations) the concentration of pantothenic acid, riboflavin and biotin was not higher than in normal milk (NorP-milk). However, the concentration of cobalamin was exceptionally high.

Little is known at present about the microbial strains in the rumen of the test cows. As mentioned in Section II.C., a large number of bacteria and the absence of protozoa are characteristic of the rumen population of O-cows, whereas the rumen population of ULP-cows resembles that of NorP-cows. The total bacterial count of the O-rumen is considerably higher than that of the ULP- or NorP-rumens.

Apparently the rumen population of cows fed urea consists largely of ammonia-consuming strains although the presence of bacteria with more complicated nutritional requirements has been demonstrated[146].

The high concentration of riboflavin, pantothenic acid and biotin in O-milk is due probably both to the increased numbers of bacteria in the O-rumen and to a vigorous synthesis of these vitamins by the ammonia-consuming bacteria. The role of protozoa in ruminal vitamin synthesis is generally considered to be negligible[37,89] but their absence might have the effect of reducing the demand on the B-vitamin pool in the rumen.

The reason for the high concentration of cobalamin in ULP-milk cannot be easily explained on the basis of microbial counts, since the cobalamin concentration in NorP-milk is between those of O- and ULP-milks whereas the microbial composition of the ULP-rumen falls between those of O- and NorP-rumens being, however, considerably nearer to that of the NorP-rumen. The cause of the high concentration of cobalamin appears to be connected with the complicated symbiotic relationships of rumen micro-

organisms. In addition, it should be kept in mind that cobalamin has not been found to occur in plants[46,70] and thus is present in the feed of the cow only when fermented fodders are given.

There was great variation with regard to the B-vitamin concentration in milk of individual cows within the same group. These variations could not be correlated *e.g.* with the differences in feeding, where such existed. For instance, in the ULP-group the conventional low-protein feed in the diet of cows Kila and Lelo (Table 13) was cereals whereas in the diet of the cow Lila (Table 14) it was potatoes. Comparison of the vitamin concentrarion in the milk of Kila and Lelo with that of Lila (Table 21) showed that, with regard to the vitamin pattern of the milk, cows Lelo and Lila could be considered similar rather than Lelo and Kila. Also in the O-group, where the feed of one cow differed from that of another only slightly, variations occurred. For example, the concentration of riboflavin in the milk of cow Aino was about twice that in the milk of cow Nairu (308 and 161 μg/100 ml respectively), and the concentration of pantothenic acid in milks of cows Aino, Eiru, Jairu and Oona was around 1000 μg/100 ml (mean 1014) wheras in the milk of cow Metta it was 656 μg/100 ml (Table 21). The differences within one group seemed to be merely a reflection of individual characteristics.

For the extraction of B-vitamins from milk different methods were used. Thiamine, riboflavin and nicotinic acid were in general extracted by the same method (dilute acid/diastase), although other methods also were tested. At the beginning of the investigation biotin and pyridoxine also were extracted by the same method. However, since the internal error in both biotin and pyridoxine assays tended to be large, vigorous acid extraction was used for biotin, pyridoxine and also nicotinic acid assays. The results obtained with this method were quite satisfactory in the case of biotin and nicotinic acid. In contrast the method was not suitable for the assay of pyridoxine, since it apparently made part of the pyridoxine unavailable for *N. sitophila*. The usual method for pantothenic acid extraction, *i.e.* digestion with intestinal phosphatase and chicken liver enzyme, could be replaced by digestion with chicken pancreas enzyme. The latter, initially designated for folate extraction, was simpler in practice than the former and gave similar results. For cobalamin assays milk was extracted by papain digestion in the presence of cyanide and ascorbic acid. Deoxyribosides to which *L. leichmannii* responds were not present, at least in interfering concentrations, in extracts prepared in this way.

Only folate was determined with two organisms, namely *S. faecalis* and *L. casei*. The latter was found to be superior to the former. As far as is known, *L. casei* is the only organism which responds to N^5-methylfolates, the main

folate in blood and liver[153]. According to the author's results methylfolates seem to be the main form of folate also in milk.

The methodology has been discussed more thoroughly in connection with the evaluation of the methods in Section D.1.

V. SUMMARY

In the present work the concentration of B-vitamins in milk was investigated in order to elucidate whether the B-vitamin level in milk is dependent on an external source of these vitamins. Cows were fed: a) rations of known chemical composition with urea and ammonium salts as the sole source of nitrogen and devoid of B-vitamins (O-feed), b) rations of low-protein feedstuffs with urea as the main nitrogen source and containing unknown amounts of B-vitamins (ULP-feed) or c) conventional rations also with unknown B-vitamin content (NorP-feed).

Microbiological methods were used. In addition, later a colorimetric method was used in riboflavin analyses.

Thiamine was estimated with *L. fermenti* in milk extracts prepared by dilute acid extraction with a subsequent digestion with diastase or diastase and papain. The mean values obtained were 46, 46 and 42 μg/100 ml respectively for O-, ULP- and NorP-milks. There was no statistical difference between the three milk groups.

Riboflavin was estimated with *L. casei* in the milk extracts prepared in the same way as for thiamine assay. During the last year of the investigation, a colorimetric method was used in riboflavin analyses. The mean levels of riboflavin were 284 and 223 μg/100 ml O-milk, 206 and 173 μg/100 ml ULP-milk and 201 and 164 μg/100 ml NorP-milk with the microbiological and colorimetric method respectively. With both methods the O-milk level was higher than that of the other two milks, the difference being significant with the microbiological method and highly significant with the colorimetric method. There was no statistical difference between ULP- and NorP-milks.

Nicotinic acid was estimated with *L. plantarum* in the same milk extracts as those for thiamine assay, or in extracts prepared by autoclaving with 6 N sulphuric acid for 1 hour. The mean concentrations were 191 μg/100 ml O-milk, 174 μg/100 ml ULP-milk and 173 μg/100 ml NorP-milk. There was no statistical difference between the three milk groups. All three extraction methods gave the same values. Extraction of O-milk with dilute acid without enzyme digestion gave a value of 95 μg/100 ml.

Pantothenic acid was estimated with *L. plantarum* in extracts prepared by digestion with intestinal phosphatase and chicken liver enzyme. The

difference in pantothenic acid concentration between O-milk and the other two milks was highly significant, the mean concentrations being 982 μg/100 ml O-milk, 349 μg/100 ml ULP-milk and 443 μg/100 ml NorP-milk. There was no significant difference between the ULP- and NorP-milk values. Extraction of pantothenic acid from milk by chicken pancreas digestion was as effective as the use of intestinal phosphatase and chicken liver enzyme.

Pyridoxine was estimated with *N. sitophila* in extracts prepared by mild acid extraction with a subsequent diastase digestion, or by autoclaving with 6 N sulphuric acid for 1 hour. The concentrations obtained with the first method were 52 μg/100 ml O-milk and 50 μg/100 ml NorP-milk and with the second method 33 μg/100 ml O-milk and 30 μg/100 ml ULP-milk. There was no significant difference between the milk groups in values obtained with the same method, but there was a marked difference in values obtained with different methods. Extraction with 6 N sulphuric acid for 1 hour was thought to be too vigorous for pyridoxine estimation.

Biotin was estimated with *L. plantarum* in the same milk extracts which were used for pyridoxine assays. The dilute acid/diastase method used at the beginning of the investigation gave mean concentrations of 3.8 and 2.9 μg/100 ml, respectively, for O- and NorP-milk, with no significant difference. With the second extraction method the mean concentration of biotin was 8.5, 1.5 and 1.6 μg/100 ml, respectively, for O-, ULP- and NorP-milks. The difference between O- and the other two milks was highly significant. The increased concentration of biotin in O-milk as compared with the earlier findings was thought to be due to changes in ruminal population at the later stage of the feeding experiments.

Folic acid was extracted from milk by autoclaving with water or by digestion with a chicken pancreas preparation and estimated either with *S. faecalis* or *L. casei*. With *S. faecalis* there was no difference between values obtained with the two extraction methods, the mean concentration of folate being 2.6, 2.1 and 3.2 μg/100 ml respectively for O-, ULP- and NorP-milk. There was no statistical difference between the three milk groups. With *L. casei* the water extraction method gave slightly lower values than the chicken pancreas digestion method; therefore the latter was used generally. The concentration of folate was 36, 31 and 32 μg/100 ml respectively for O-, ULP- and NorP-milks. There were no significant differences between the three milk groups. The chicken pancreas/L. casei method was considered to be superior for estimating milk folate.

Cobalamin was estimated with *L. leichmannii* in extracts prepared by papain digestion. The mean concentrations of cobalamin were 394, 1295 and 621 mμg/100 ml respectively for O-, ULP- and NorP-milk. There was a highly significant difference between ULP-milk and the other two milks and an almost significant difference between O- and NorP-milks.

It is concluded that a ration of known chemical composition with urea and ammonium salts as the sole source of nitrogen and devoid of B-vitamins (O-feed) caused an increase in the concentrations of pantothenic acid, biotin and riboflavin in milk while the concentrations of thiamine, nicotinic acid, pyridoxine, folic acid and cobalamin were not affected. The addition of urea to a low-protein ration (ULP-feed) caused a 2- to 3-fold increase in the concentration of cobalamin, whereas the concentration of the other B-vitamins was not affected. Evidently the rumen populations adapted to use the O-feed was able to satisfy the B-vitamin requirements of the cows.

Various aspects of the microbiological methodology are discussed.

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